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STUDIES OF THE PHYSIOLOGICAL ANATOMY OF THE STRAWBERRY[1]

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INTRODUCTION

The material herewith presented is the result of a study of the physiological anatomy of the strawberry (Fragaria spp.) undertaken in connection with the work in breeding this genus which is being carried on by George M. Darrow, of the Bureau of Plant Industry. The present paper is based on the anatomy of the varieties known as "Howard 17" and "Progressive" (both probably descended from crosses between *Fragaria chiloensis* and *F. virginiana*), supplemented as regards certain features particularly pertinent to the problems in mind, by comparison with eight other characteristic species and varieties.

Some of the specific questions which have presented themselves for consideration are: (1) The runner being such a slender, flexible, exposed organ, what are the structural features which enable it to carry sufficient water and nutrients for the maintenance of many runner plants with their large leaf areas before these have established direct contact with the soil? (2) How long is a runner morphologically; is it composed of one, two, or several internodes? (3) Why does the first runner from a runner plant develop so much more rapidly than do the later ones? (4) Why are plants having only the large, white roots, so commonly observed, less likely to succeed on transplantation than those without such roots? (5) What is the structure which enables water and nutrients to move freely from one side of the plant to the other?

Except for the incidental references contained in the general works of Gérard (*2*),[3] Reinke (*4*), Trécul (*7*), Solereder (*5*), and Van Tieghem and Douliot (*8*), no information concerning the anatomy of the strawberry is available, as far as could be learned.

MATERIALS AND METHODS

The plants used in these studies were obtained from the breeding plots of the Bureau of Plant Industry at the Bell Horticultural Field Station at Glenn Dale, Md., and the preparations were made in the botanical laboratories of Johns Hopkins University at Baltimore. Most of the sections and other preparations were made from plants collected in early November and fixed in medium chromacetic fixative. These were supplemented with fresh materials for dissection and for

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[2] Acknowledgment is given to those who have aided in this work, particularly to George M. Darrow for the original problem and to D. S. Johnson, professor of botany in Johns Hopkins University, for the freedom of the Johns Hopkins botanical laboratory, for valuable suggestions in the conduct of the investigation, and for help in correcting the manuscript.

[3] Reference is made by number (italic) to "Literature cited," p. 492.

sectioning on the freezing microtome, collected from time to time at the same station.

A number of stains were tried and Ehrlich's haematoxylin was finally chosen. In addition, some root sections were stained in Haidenhain's and Delafield's haematoxylins, methylene blue, and Flemming's triple stains, and some small tissue fragments in safranine-malachite green. Ehrlich's haematoxylin did not give a very satisfactory stain, apparently because of the large amounts of tannin and free acids present in the material, but it gave better results than any other stain tried. At first it was thought that the difficulty was due to the fact that the chromic acid of the fixative was insufficiently washed out, although material was washed for 48 hours in running water. It was found, however, that sections cut from living material on the freezing microtome gave similar results. Hence, it was concluded that the trouble lay in the material itself and not in the treatment. Great difficulty was encountered in cutting embedded material owing to the presence of large numbers of crystals, presumably of calcium oxalate, in the parenchyma of both the crown and the veins of the leaf; to the great lignification of the vascular elements, particularly in the fibrous roots; to the abundance on the above-ground portions of greatly thickened, probably silicified hairs (pl. 1, C and D, and pl. 2, D); and to the difficulty in removing all sand lodged between the closely packed adventive roots. The series from which the leaf traces and much of the crown anatomy were studied were therefore treated for 48 hours in 52 per cent hydrofluoric acid and infiltrated by very gradual steps either in celloidin or paraffin. Paraffin sections of crown vascular elements were cut 3μ in thickness; roots, runners, leaves, etc., 7μ; sections in paraffin for the gross anatomy of the crown, 15μ; and celloidin series for reconstruction, 40μ. Series of crowns more than 2 years old have not yet been obtained, this being left for future work. Satisfactory "dissections" of whole crowns have been obtained by boiling in caustic solution and later washing away the parenchyma with a jet of water.

THE RUNNER

In commercial propagation the vast majority of strawberry plants come from runners and not from seed. The "runner" is a very much elongated diageotropic [4] stem and can easily be distinguished from the large adventive roots by its hollow cylinder of vascular tissue, which is covered by a thick layer of cortex and which surrounds a very large parenchymatous medulla. (Fig. 1.) The vascular cylinder has an extensive primary xylem composed of exceedingly large vessels arranged in distinct but closely approximated bundles. Each of these bundles is made up of wedges of primary xylem separated by quite irregular medullary rays of one to four cells in tangential thickness. (Pl. 3, E.) This primary xylem is the chief functional portion of the xylem throughout the comparatively short life of the runner. Outside this in old runners is found a considerable amount of secondary xylem made up of much smaller, more heavily lignified tracheids. The whole is admirably adapted

[4] The physiological reactions of these runners have not been studied as yet, as far as is known. It is therefore uncertain whether the horizontal position is merely due to the runner being too weak anatomically to support itself in an erect position or whether it is the result of an active tropism.

for carrying the large amounts of food and water required for the complete establishment of the daughter plant. The medulla is made up of large, loosely packed, thin-walled cells quite different from the storage pith of the crown. The phloem is well developed. (Pl. 3, E.) The outer portion of the pericycle of the runner (pl. 3, C)[5] shows a less extensive tangential multiplication than is characteristic of that of the adventive roots and of the crown proper.

The runner has greatly elongated internodes which are covered with short unicellular hairs interspersed with numerous multicellular glandular ones. The first node bears a solitary leaf which often remains rudimentary. Each such leaf has in its axil a bud potentially capable of development as a runner or crown. The arrangement of bundles is particularly clear at the nodes of a runner. The base of the leaf encircles the stem in an oblique spiral, one stipule being much shorter than the other. The three bundle traces (fig. 1) are located at about equal distances from each other around the stem. The middle one rises highest; the right-hand one is usually somewhat lower down, and the left-hand one is lowest of all. This order is characteristic of the leaf traces thoughout the entire plant. Where the nodes are close together the first indication of separation of the traces for the next higher leaf can be observed, one close to each of the traces here mentioned (fig. 1), but the cylinder is broken only for a short distance, the leaf-bundle gap formed being quite short. The two lateral leaf traces swing toward the central one in the petiole sheath, and from them arise the several stipular bundles.

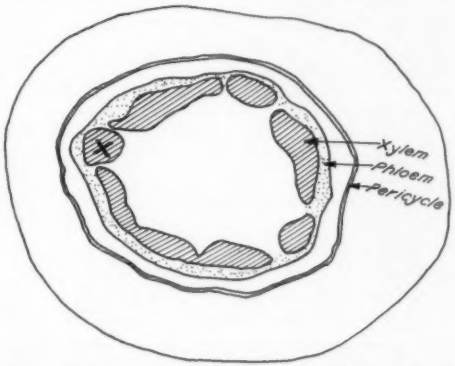


FIG. 1.—Transverse section of a runner near the node. The bundle at X is the median leaf trace of the leaf next above the position of this section. The xylem is well developed. × 80

After the runner has grown diagetropically for several centimeters its tip becomes negatively geotropic, turning up just beyond the second node and undergoing great thickening. It also puts forth adventive roots, and thus soon becomes established as a daughter crown.

The leaf which subtends each such crown remains attached often until its base is completely buried in the cortical parenchyma of crown and root. Its axillary bud sometimes remains dormant and is likewise buried with it in the parenchyma of the daughter crown. More often, however, this axillary bud continues to develop as a new runner. Since the growing tip of the primary runner, now that of the crown, has turned upward (pl. 1, B), the tip of this secondary run-

[5] Van Tieghem and Douliot (*8*) state that in the strawberry the pericycle of the roots may be of two or three layers, but that the innermost layers of the cortex undergo proliferation to form a periderm. They figure the endodermis as lying between two series of rectangular cells. What is termed "pericycle" in the present paper has not been tested microchemically for cutin, but there is no appearance of a distinct layer where, according to these writers, the endodermis should lie.

ner has all the appearance of being a continuation of the parent one. This appearance is enhanced by an anatomical peculiarity. The central trace of the leaf does not separate from the vascular cylinder of the runner below the origin of the bud, but remains as part of the continuous cylinder well up on the outer side of the axillary bud, above its insertion into the base of the crown. A longitudinal section of the runner at this point through the median leaf trace would therefore show the leaf apparently arising from this bud as from a main axis. (Fig. 2.) It is only the insertion of the other two traces into the daughter crown that proves this bud to be really an axillary and not a terminal one. (Figs. 3 and 4.)

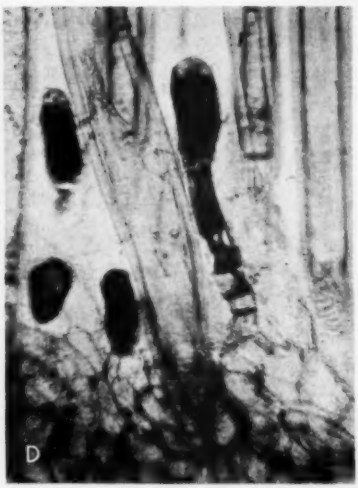
FIG. 2.—Longitudinal section of the vascular system of the tip of a runner, showing relation of crown, subtending bud, and leaf. The median trace of the leaf arises from the bud as if froma primary axis. The position of the lateral traces (figs. 3 and 4) shows the crown to be the true primary axis, the bud being axillary

THE ROOTS

The primary runner does not give off roots below the leaf subtending the secondary runner. These arise solely from the crown proper. The vascular system of the very base of the crown is a continuous inverted cone broken only by the two lateral leaf traces and, in those cases where a secondary runner develops, by the branch trace leading to it. (Pl. 3, D.) If the axillary bud remains dormant, no branch trace is formed above it in the maturing vascular cone. If, however, this bud continues growth, as often happens, a decided gap is formed in the vascular cone of the crown. This gap does not, however, extend far up the crown. The adventive roots arise directly from the pericycle just outside the surface of this vascular cone, or in older crowns from just outside its ramified vascular strands. They arise by tangential division of the cells of the multiple pericycle, accompanied by a differentiation of the tissues intervening between this and the vascular elements of the crown, into xylem and phloem elements. The root trace leaves no opening in the parent cone. The contact is made by individual groups of xylem cells of the crown. These roots are established on the younger portion of the crown, which, while its vascular system has a certain amount of mature xylem, is by no means completely formed. In most cases these roots do not undergo secondary thickening, and hence their xylem elements do not connect with the later secondary xylem developed by the crown. Sometimes, however, the roots do become secondarily thickened. In such cases, as new xylem is laid down by the parent cylinderof the crown it is formed

FIG. 3.—Longitudinal section of the vascular system of the tip of a runner, showing a lateral trace arising from the crown

FIG. 4.—Diagrammatic representation of the arrangement of the three traces of the leaf which subtends a runner crown

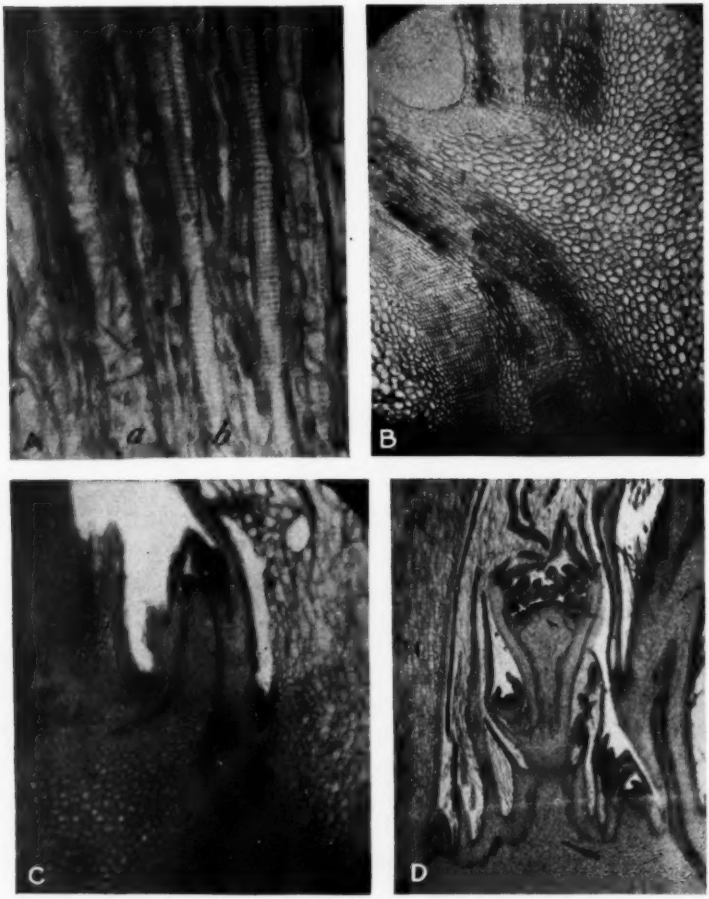


A.—Dissection of old crown to show the vascular skeleton. The basal cone has been cut away and the youngest portions dissolved, leaving only the mature secondary xylem. Note spiral arrangement of the three main strands which are tied across by numerous narrower bands. The latter are also spirally placed but in the opposite turn. × 3.75

B.—Runner tip with the beginning of a new crown, before the formation of roots. The terminal bud has turned upward. Note the reflexed nodal leaf from the axil of which the runner will be continued. × 2½

C.—Longitudinal section of hairs from the upper part of the crown. The bases of the hairs are deeply embedded in the outer layer of cortical parenchyma. × 375

D.—The same with accompanying multicellular glandular hairs. × 750

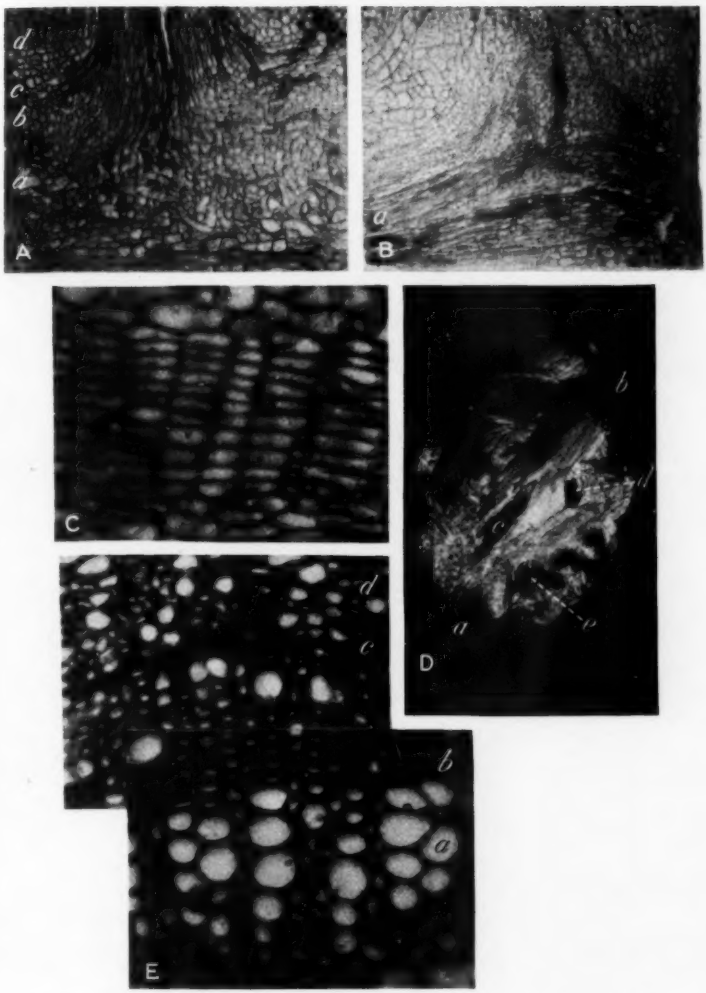


A.—Radial longitudinal section of a young xylem strand, showing the annular (*a*) and spiral (*b*) vessels of the primary xylem. × 350

B.—Radial longitudinal section of a crown, showing a leaf-trace gap. The vascular tissue of the crown is broken off squarely above the gap. × 88

C.—Radial longitudinal section of a crown, showing a branch gap. The vascular cylinder of the crown is broken only at the center of the daughter cylinder, no external gap being formed. × 88

D.—Longitudinal section of the apex of a mature crown, showing the vascularization of young inflorescence. Note the protective hairs. × 21



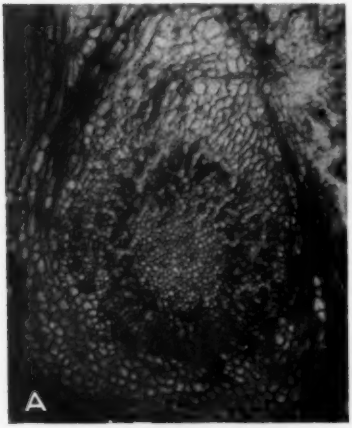
A.—Longitudinal section of a crown, showing connection of the various tissues of a young adventive root with those of the crown: *a*, Xylem; *b*, cambium; *c*, phloem; *d*, pericycle. Note particularly the continuation of the many-layered pericycle of the crown into the root. × 125

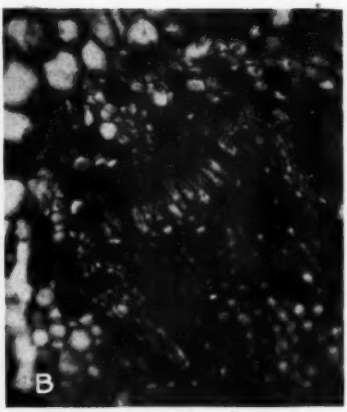
B.—Section as above but showing particularly the phloem (*a*). × 125

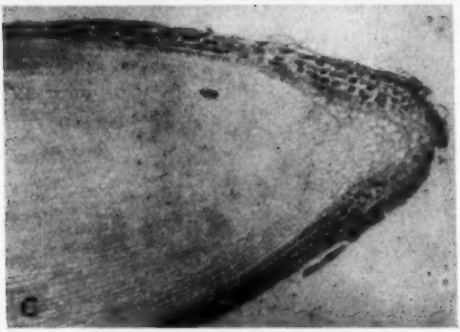
C.—Transverse section of runner, showing the many-layered pericycle or periderm. Note the large cells of the extrapericyclic cortex above. The parenchyma between the pericycle and the phloem is made up of much smaller cells. × 250

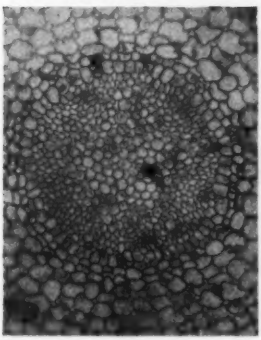
D.—The vascular system of the base of a young crown prepared by maceration. The photograph is taken from below. The parent runner is at *a* and the secondary runner at *b*. Note the bundle gap (*c*) of the medium bundle of the leaf subtending the secondary runner. The runner or branch gap (*d*) above is noticeably small in spite of the considerable development of the runner. Adventive roots are to be seen at the sides. The gap at *e* is probably that of one of the lateral bundles of the leaf. × 7½

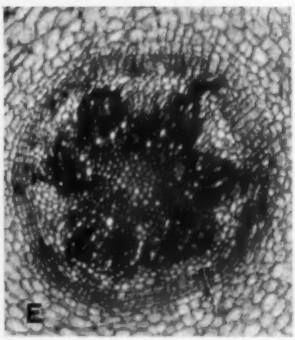
E.—Transverse section of the vascular tissue of a runner, showing primary (*a*) and secondary (*b*) xylem, cambium (*c*), and phloem (*d*). Note the size of the conducting vessels in the primary xylem. × 250











A.—Transverse section of adventive root having an apparently polyarch stele and large medulla. The section is taken just at the level of the phloem in the crown so that the pericycle is not visible. × 175

B.—Transverse section of an irregularly pentarch root, showing the formation of an interfascicular cambium initiating secondary thickening. Note that here the cells of the medulla are also thickened to form a pseudovascular stele. These cells are, however, not greatly elongated as are the cells at the center of the steles of the fibrous lateral roots. Such cases are rare. × 175

C.—Longitudinal section of the growing point of an adventive root. The various elements do not begin to differentiate until well back of the growing point. Compare with Plate 5, C. × 70

D.—Transverse section of an adventive root taken just back of the growing point. The stele is here pentarch from the beginning. × 350

E.—Transverse section from an older similar root taken near its insertion into the crown. The stele is hexarch. Note the multiple pericycle and the relatively distant phloem strands. × 175

chiefly above the root and is connected to its secondary xylem. A thin area is thus left in the vascular cylinder of the crown just below the point of origin of the root. These areas are never large and appear to occur only below secondarily thickened roots.

These adventive roots, which give rise to the entire root system of all except seedling plants, arise apparently from any part of the central cylinder of the crown, and are very often found actually pushing through the bases of still functioning leaves. Van Tieghem and Douliot (*8*) state that these roots can arise only at the two sides of a median leaf trace. The xylem strand is already differentiated before the root has pushed out through the cortex of the crown. (Pl. 3, A and B.) Once established, however, it pushes out very rapidly, its tissues maturing slowly so that the vascular elements beyond the surface of the parent crown are not differentiated until the root has attained almost its full diameter. (Pl. 4, C.) The vascular strands are therefore widely separated. The stelar plan of these roots appears to be exceedingly varied. It is either tetrarch, pentarch (pl. 4, D), hexarch (pl. 4, E), or in a few cases, apparently polyarch (pl. 4, A). No actual transition between these types has been observed, but from the differences noted in different sections of a single root it seems possible that they may sometimes change from one to another in the same root. It is, however, perhaps significant that both tetrarch and pentarch steles have been found at the first visible differentiation behind the growing point. Hence, steles of more than four strands do not necessarily arise by transition from this type, but apparently are formed as such from the beginning. The steles of these roots (at least in the material at hand) are never heavily lignified.[6] The phloem is well developed (pl. 4, E) and the pericycle usually many layered (pl. 4, E). The endodermis is distinguishable only in the very early stages. (See footnote 5.) Transition to a cylindrical stele by the formation of an interfascicular cambium does occur, but it is not the rule. (Pl. 4, B.) The cortical and medullary parenchyma are extensive but thin walled, and are usually almost devoid of stored starch or other observable materials. There are, in the older portions, large numbers of cells filled with what appears to be tannin. (Pl. 4, B.) The contents of these have not yet been tested microchemically, however, so that their exact nature is uncertain.

These adventive roots push out very rapidly and, as is shown by examination of the root systems in older plants, may reach a length of 4 or 5 inches. (Pl. 6.) Darrow has found instances in which these roots developed to a length of 10 inches without branch or taper. He also reports cases in which such roots bore root hairs throughout their entire length. Such cases have, however, not occurred in the material examined in this study. In the writer's material they do not appear to form root hairs themselves but give rise to lateral branch roots which are the true absorptive organs.

These secondary roots differ markedly from the parent roots. Their mode of origin is essentially that described by Van Tieghem and Douliot (*8*) for rootlets of Fragaria. They have, however, a decidedly different stelar plan from the parent type. The primary

[6] A few cases of development of interfascicular cambium have been observed in which secondary thickening took place. Such secondary thickening was accompanied by a lignification of the medullary parenchyma cells to form a pseudovascular stele. These cells, however, were not elongated and were unmistakably different from the central vascular strands of the fibrous roots. (See pl. 4, B.)

vascular structure is already differentiated at a point close behind the root apex. The marked thickening ultimately shown by these roots is therefore chiefly secondary. It is often possible to distinguish the first spirals of vessels in the root at a point less than its own diameter from the apical meristem, and indeed at a point which is still covered by the short rootcap. (Pl. 5, C.) The vascular bundle in these roots varies from a minute strand having a single large central vessel (fig. 5) which, but for the two lateral rays of small vessels, one on either side, might persuade one that he was dealing with a monarch stele, through diarch (pl. 5, D) and triarch (pl. 5, E) to the tetrarch type (pl. 5, A). No examples of more than four xylem rays were found in these branch roots. The diarch and tetrarch are by far the most common. Roots have been examined for this feature in four species and five varieties of Fragaria with like results. These vascular strands are heavily lignified and completely fill the center of the root. (Pl. 5, A.) There is no medulla whatever here, such as is present in the adventive roots, and the lignification progresses outward until the xylem portion of the stele consists of a solid woody strand. The phloem is well developed. (Pl. 5, A.) The endodermis, unlike that of the parent root, has greatly thickened walls (pl. 5, A and D), which perhaps serve as a protection against the fungi that are usually present in the cortex. The pericycle shows no tangential multiplication whatever such as is found in the parent type, remaining as a single layer of cells. Outside of the endodermis the cells of the cortex are filled with starch and certain of them harbor a fungus, which may be of a mycorrhizal character.[7] These roots bear numerous root hairs which are in no way peculiar.

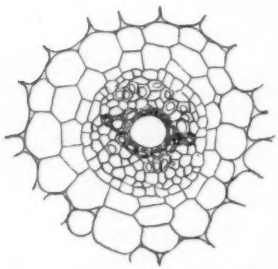


FIG. 5.—Transverse section of the stele of a fibrous root of *Fragaria elatior* which is diarch but has a single large central vessel. × 250

These two sets of roots make up the very extensive and efficient absorbing system of the strawberry crown.

THE CROWN

The crown is at first a short fusiform body. It may later, by apical growth, become cylindrical and eventually fork into two or more divisions. The subterranean portion is covered with adventive roots. Aboveground it is clothed with leaves, the base of each of which closely encircles it for some three-fifths of its circumference. These leaf bases are closely imbricated and are provided with three distinct vascular strands each. The stipules are well developed and foliaceous. The petiole at its base is semicircular in cross section. The phyllotaxy is of the 2/5 type with a very much shortened spiral. Nowhere except in the runner does this spiral become much elongated.

As the leaves die their bases remain as dry, blackened scales. The three vascular strands persist long after the softer tissues have decayed, so that it proved quite easy to work out the overlapping of

[7] WHITE, P. R. MYCORRHIZA AS A POSSIBLE DETERMINING FACTOR IN THE DISTRIBUTION OF THE STRAWBERRY. [Unpublished manuscript.]

these bundles from such plants—much easier, in fact, than from serial sections. Working from below upward (figs. 6 and 7), the left-hand bundle of leaf No. 4 is just above the median bundle of leaf No. 1, while the right-hand bundle of leaf No. 5 lies above and on the same orthostichy as these two. The median bundle of leaf No. 6 lies directly above but a considerable distance from the median bundle of No. 1, so that two consecutive median bundles occupy positions in the order of imbrication, 2/5 of the stem circumference apart. In this way, two turns around the stem and five leaves are required between two leaves on the same orthostichy. The leaf traces themselves do not leave wide gaps (pl. 2, B), but above the branch trace of the axillary bud which is present over each median leaf trace there may be left a considerable gap (pl. 2, C), particularly when the bud develops to an inflorescence or a runner. The branch gap often becomes completely confluent with that of its subtending median leaf trace. It is, however, of later origin, the leaf trace being completely distinct before any indication of a branch stele becomes evident. (Fig. 8.)

These leaf bases, and more particularly the axillary buds and the structures subsequently developed from these, are densely covered with long, very much thickened, protective hairs. These hairs are unicellular, have a smooth outer surface and, except for the articulation with the stem or leaf surface, have a very small lumen as compared with their diameter. They are, however, quite thin walled at their bases. They originate as outgrowths of the cells of the epidermis, but their bases push inward so as to extend a short distance beyond the general inner limit of this tissue. (Pl. 1, C and D.) Such hairs are particularly numerous in the immediate angle between the leaf base and the stem, forming a fringe of longer hairs on the stem at the line of juncture. They are accompanied by lesser numbers of short, apparently glandular hairs, each of which is made up of several short cylindrical cells and an enlarged terminal one. (Pl. 1, D.) These glandular hairs may perhaps secrete a volatile oil, though the nature of their secretion has not actually been determined.

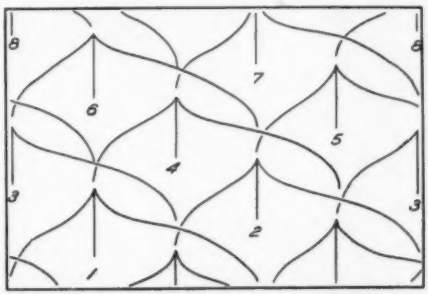


FIG. 6.—Diagrammatic representation of the arrangement of leaf traces. Since the bundles of one leaf do not connect directly into those of another, no representation has been made of the main vascular cylinder of the crown, but only of the bundles back to their connection into this cylinder

Aside from these emergences, the epidermis of the crown is made up of cells of four-angled or polygonal outline which show no apparent protective modifications. The function of protection is apparently entirely relegated to the leaf bases. No indication of suberization or cutinization was observed. Van Tieghem and Douliot (*8*) mention "liége" in speaking of the periderm, but it is uncertain as to just what they refer.

Below the epidermis in all regions of crown and roots there is a considerable primary cortex of large, quite closely packed, parenchyma-

tous cells, which in many regions, particularly the older parts of root or crown, show reticulate thickenings on the walls similar to those of the medulla and of the epidermis of the leaf. (Pl. 7, A.) No cork cambium was seen at any point, though Van Tieghem and Douliot (*8*) mention the occurrence of periderm here; and no bast fibers or other supporting tissues are present. In the entire plant, even to the leaves, the function of support seems to be served by the tracheids alone. Within the cortex, in the stele itself, the pericycle undergoes division, forming quite a large ring of thin-walled cells closely resembling those of a cambium. (Pl. 7, C.) In the maceration of crowns for the study of the vascular system the outer tissues peeled off in two layers, the first from epidermis to pericycle, the second from pericycle to cambium. The endodermis is quite indistinguishable except in the very early stages before the commencement of this division in the pericycle. Van Tieghem and Douliot (*8*) figure the endodermis as in the middle of the pericycle-periderm complex, but the writer has not been able to distinguish it in his material. Within the meristematic layers of the pericycle is a narrow region of parenchyma of somewhat smaller cells than that without. These are in part cells of inner layers of the pericycle and in part cells of the outer edges of medullary rays. The primary phloem strands are widely separated. The secondary phloem itself never becomes even a comparatively continuous cylinder except for a few layers which are broken into wedges separated by wide medullary rays. It appears to be made up of simple, thin-walled cells which are somewhat elongated and interspersed with very sparse companion cells. No sieve plates, either terminal or lateral, have been observed in any preparations, the phloem being distinguishable in longitudinal section from the parenchyma chiefly by its elongated, oblique-ended cells (pl. 7, D) and by its darker staining reaction. In a tangential section the medullary rays which pierce it are made up of large, thin-walled rounded cells accompanied by somewhat smaller cells having dense protoplasts and large nuclei like those of the xylem ray shown in Plate 7, E. There is no indication of thickening or pitting in ray cells. The cambium is of few cells in thickness and of hardly sufficient regularity to constitute a definite ring or cylinder.

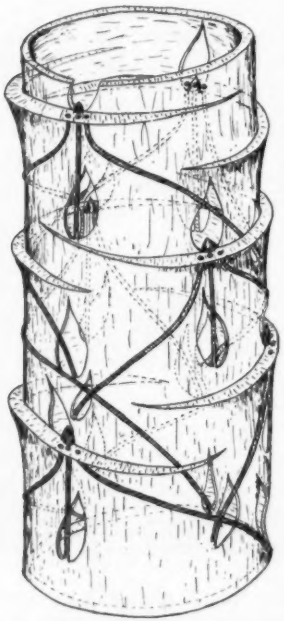
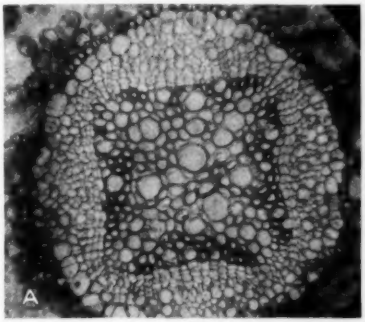
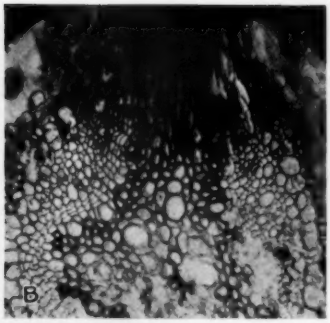
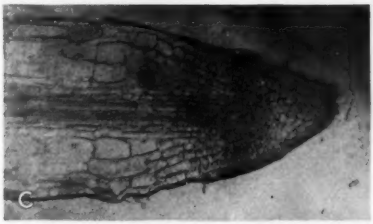


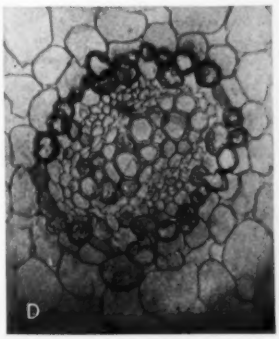
FIG. 7.—Leaf-trace arrangement, semi-diagrammatic. The bundle gaps are actually much larger than suggested by this figure. (See pl. 1, A.) Each leaf draws its water from three quite distant portions of the crown circumference

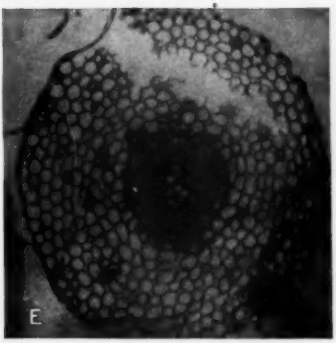
The leaf traces, as has been said, overlap in a perfectly regular manner. They do not, however, connect definitely with other traces, as is true in many herbaceous plants as well as in certain woody ones (*3*), but are fused into what, from its basal condition, we may interpret as a continuous woody cylinder broken only by the leaf and











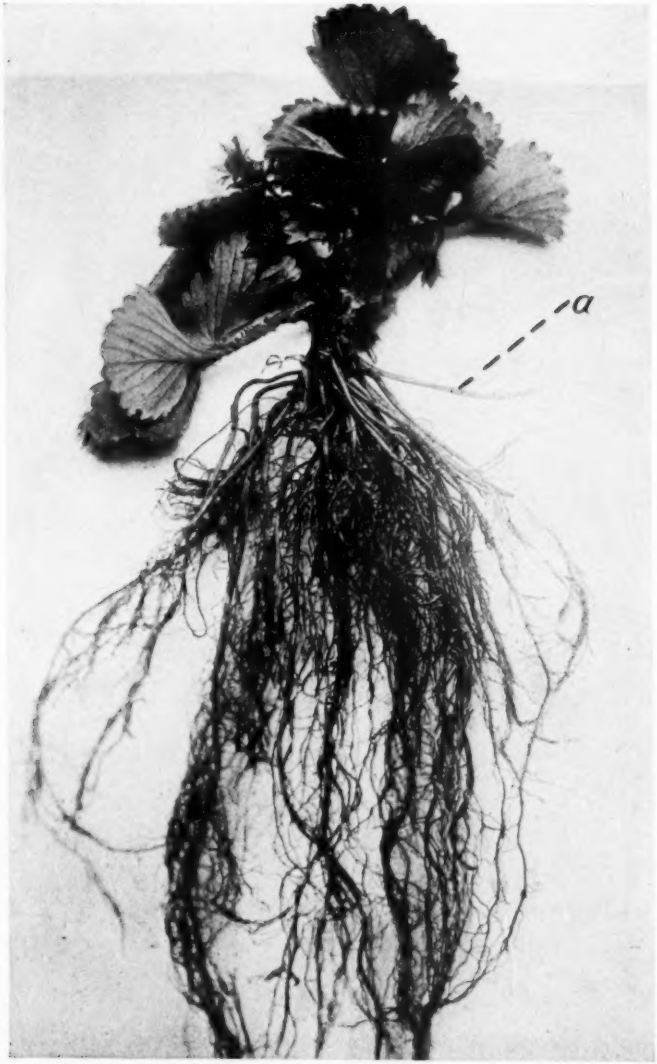
A.—Transverse section of a fibrous root having a tetrarch stele. The primary and secondary xylem are clearly distinguishable by their staining reaction. Both fascicular and interfascicular cambiums are active and the phloem is well developed. The pericycle is unmodified, whereas the endodermis is greatly thickened. The innermost cortical layers are filled with tannin. Compare with Plate 4, A, D, and E. × 160

B.—Origin of a branch root from a fibrous root such as that shown in A. × 160

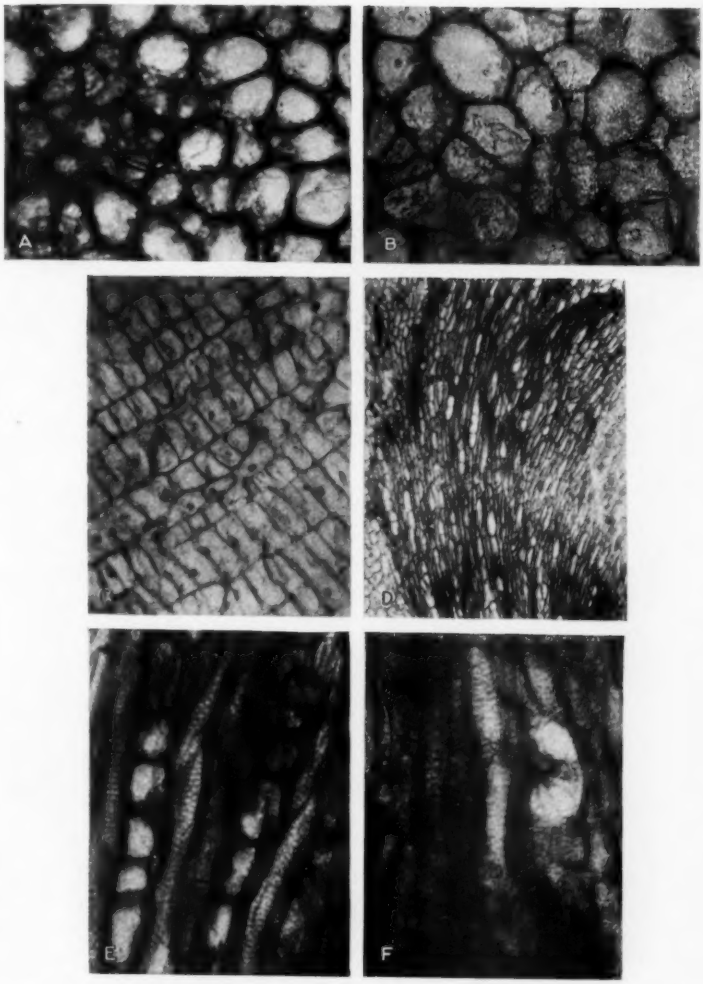
C.—Longitudinal section of the growing point of a fibrous root. The differentiation of vascular elements takes place very close to the apex. Compare with Plate 4, C. × 160

D.—Transverse section of a small fibrous root, showing the diarch stele. The endodermis and pericycle contain some tannin. × 320

E.—Transverse section of a fibrous root having a triarch stele. × 64



Entire strawberry plant. Note the stocky unbranched adventive roots (*a*) which give rise to slender fibrous ones. × ⅔



A.—Section of medullary parenchyma showing reticulate thickenings in the walls. Section taken from near the vascular cylinder. × 250

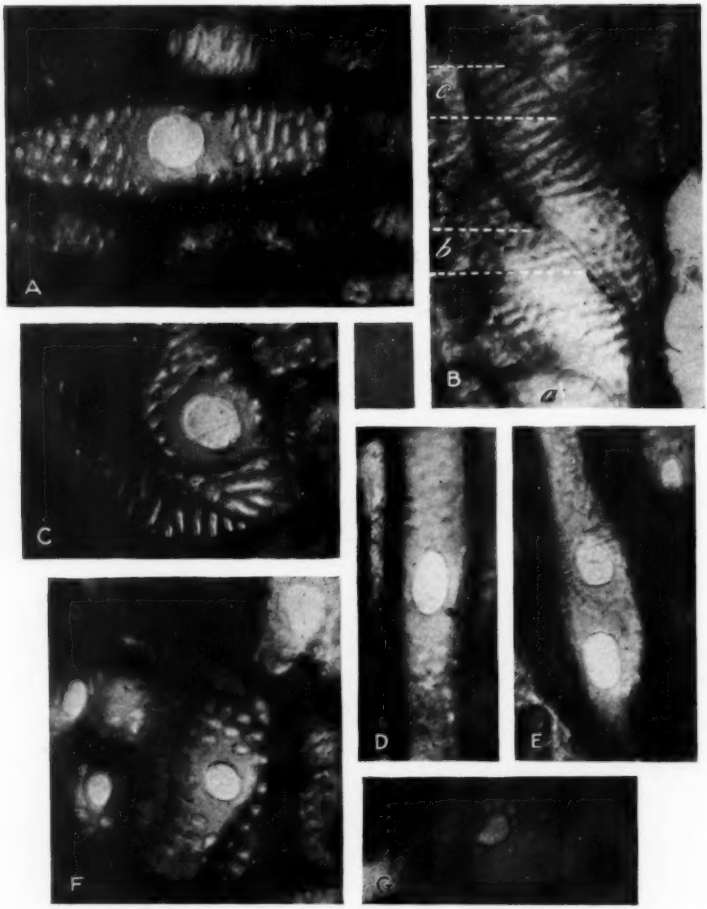
B.—The same tissue nearer the center of the crown, where such reticulations are lacking. × 250

C.—Transverse section of the multiple pericycle of the crown. × 250

D.—Tangential section of the crown in the region of the phloem, showing the oblique-ended conductive cells and the narrow, few-celled medullary rays. × 63

E.—Tangential section of crown xylem, showing detail of the medullary rays. The rays piercing the phloem are similar in structure. × 500

F.—Radial section of secondary xylem. The pores in the tracheids are clearly in the lateral walls. × 500



A.—Radial section of xylem of the crown, showing a circular pore in the lateral wall of a short tracheid. × 600

B.—Tangential section of xylem, showing pores. The three pores at *a*, *b*, and *c* connect the short tracheids, forming an oblique conducting element. × 600

C.—Note the area, immediately around the large pore, which is free from pits. Note also the scalariform thickenings. × 600

D.—An elongated pore in the center of the lateral wall of an unusually long tracheid. × 600

E.—Section showing two pores in the same lateral wall of a tracheid. × 600

F.—Section showing the relative abundance of these pores. × 600

G.—Rings of bordered pits encircling pores in this manner appear to be very rare. × 600

branch gaps. This cylinder is not made up of uniformly longitudinal tracheids or tracheae (see footnote 8, p. 490) as are those of most woody stems. The strands are not simply parted at the gaps and fused again above. Bands in which the tracheids lie, in a tangential plane, perpendicular or oblique to the long axis of the plant are the rule rather than the exception. (Pl. 1, A.) It is only rarely, even immediately below a leaf or stem gap, that one obtains true transverse sections of the elements. In many cases narrow bands of xylem traverse horizontally what would otherwise be large gaps. (Pl. 1, A.) A very complex anastomosis thus arises which provides very efficient channels for a rapid and uniform distribution of material from any point throughout the circumference of the plant. The efficiency of this system is still further increased by the presence of large numbers of pits or pores similar in form to those found in the end walls of tracheal elements of many dicotyledonous plants. These pores are, however, found not only in the end walls but also in great numbers in the radial lateral walls of the tracheids, together with bordered or simple pits of the ordinary type. (Pl. 7, F.) They have not been found in the tangential walls of these elements. These pores are usually of a diameter of about two-thirds that of the cell lumen and show no trace of a middle lamella, even in sections cut from living material. (Pl. 8, B.) The pores are in most cases quite circular, but the occasional irregularities of outline and the rare occurrence of crossbars suggest the possible origin of the large pores by the fusion of smaller ones. The rarity of such irregularities and the entire absence of definite intermediate stages even in the xylem of very young crowns make it seem much more probable that the pore is due to the absence from the first of any secondary deposition of material on this area of the cell membrane and a breaking down of the middle lamella itself. Each pore is usually found in the center of an area of cell wall free from pits (pl. 8, A, C, D, and F), but a few cases were observed in which a distinct ring of bordered pits was present around such a pore (pl. 8, G). Cells have been found with more than one such pore in the same side (pl. 8, E), and others with pores in the opposite walls. Long series of cells have also been observed, in tangential longitudinal section, where pores in the lateral walls connect the whole series into one oblique continuous passage. (Pl. 8, B.) In this way, tangential circulation in the crown is not only provided by the peculiar arrangement of the vascular strands themselves and by the interweaving of the leaf traces, but a

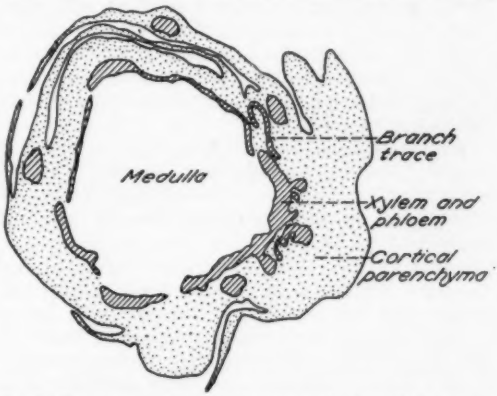


FIG. 8.—Transverse section of entire crown, showing relative proportions of the tissues. Compare with Figure 1 (runner). × 10

continuous circulatory system is provided, both longitudinally and tangentially within each individual xylem strand of the crown.

The vascular system of the strawberry plant (xylem at least) may be compared to a double-walled Sach's bell jar, in which universal diffusion of fluid materials in all directions is interrupted only by the leaf gaps and branch gaps. These pores are found in the xylem of all of the older portions of the crown, particularly in the regions of root origins. A cursory examination showed them to be present in the species *Fragaria virginiana*, *F. chiloensis*, *F. elatior*, *F. nilgerrensis*, and *F. vesca* horticultural var. Monstrueux Caemeux, and in the varieties known as Howard 17, Progressive, Dunlap, Klondike, and Rockhill. It is to be presumed, therefore, that this feature is of general occurrence throughout the genus. This condition seems clearly to explain the physiological phenomenon observed by growers that, unlike most plants, injury to or severing of the roots on one side of a crown has no more serious effect on that side than on other parts of the plant. It can be said without a possibility of doubt that no direct connection exists here between individual branch and root such as appears, from the work of Auchter (*1*), to occur in the apple. The strawberry plant has one continuous tangentially interlocking water-conducting system rather than a group of essentially distinct systems.

As is to be expected in a system with these peculiarities, true vessels [8] are not abundant. The tracheids of the xylem are exceedingly short and stout. The primary xylem, it is true, is made up of a few large annular vessels and some spiral ones (pl. 2, A) which are quite long. Such vessels, however, appear to be present only in the primary xylem. When secondary xylem is laid down, it is in the form of short tracheids only. No true vessels are formed. The pitting varies with the position of the elements from the simple scalariform reticulations of the earliest portions to the complex bordered pits with narrow, oblique slit openings of the mature type. All gradations exist between these two. The medullary rays, like those of the phloem, are but few cells thick in longitudinal and tangential dimensions, but the cells are quite large. They appear not to be provided with pits of any kind. (Pl. 7, E.)

Within the xylem the central medulla is made up of large cells. The walls of these show distinct reticulate thickenings leaving irregular pits between. (Pl. 7, A and B.) These pits become more prominent from the center of the medulla outward, suggesting that they may possibly serve to allow movement of stored food in the neighborhood of the xylem elements. Similar reticulations are also present in the cortical parenchyma of the crown and sometimes in the parenchyma of the adventive roots. Starch has not been observed in these cells and the nature of their contents has not yet been determined. It is perhaps noteworthy that the same type of reticulation is to be found in the lateral walls of the cells of the upper epidermis of the leaf. Among all these medullary cells are large numbers of crystals, probably of calcium oxalate. Such crystals are also found in the cortical parenchyma and in the tissues of the leaves adjacent to the bundles.

[8] Strasburger's (*6*) text defines tracheae as "wider or narrower tubes formed from a number of cells by the disappearance of their end walls," while tracheids are defined as "single cells with pointed ends, and are as a rule of narrow diameter. Their walls bear peculiar pits." According to these definitions no true vessels are formed in the secondary xylem of the strawberry. The structures herein described certainly represent a transition type between tracheids and tracheae, but they resemble the former much more closely than they do the latter.

DISCUSSION

The studies reported here have shown certain structural characteristics of the strawberry which are fundamental to an understanding of the growth and development of this plant. The runners with their long internodes are true stems, although their vascular structure differs markedly from that of the short internoded crowns. These differences are closely correlated with the essential differences in function of the two types. Although the xylem is extensive in both types of stems, that of the runner is composed of large, open, true vessels arranged in parallel bundles which allow free longitudinal movement of the large quantities of water and nutrients necessary for the maintenance of the runner plant and its dependent plants. In the crown, on the other hand, the entire vascular cylinder is made up of a network of short, anastomosing bundles which provide an efficient means of rapid transfer of water and solutes across the stem. Moreover, these bundles themselves, instead of being made up of long vessels, are composed of short tracheids provided with numerous large lateral and terminal pores which further increase the efficiency of cross as well as longitudinal transfer of water. Such a crown structure makes it possible for plants which have had most of their roots cut by a hoe or insect, or destroyed by fungi, to supply all leaves, runners, and other parts uniformly with water and nutrients. Broadly speaking, moisture and nutrients supplied to one side of a row of plants can be assumed to supply both sides of the plant uniformly, in striking contrast to most woody plants. Reduction of any part of the root system affects the plant as a whole rather than one side alone.

The runner is commonly two nodes in length and is not continuous beyond its first daughter crown, the runner being continued by what is really the bud from the axil of its first leaf. The manner of insertion of the lateral bundle traces of this leaf establish this point. The bud in the axil of this first leaf, however, owing to the bending up of the tip of the runner, is placed so nearly in direct line with the vascular bundles of the parent runner that it is in an especially favored position as regards its supply of water and nutrients. Hence its development is commonly far more rapid than is the development of any of the buds in the axils of later leaves on the same plant.

Marked differences in the arrangement, development, and structure of the vascular tissues of the large adventive roots which arise from the crown, as compared with the small fibrous ones, together with the paucity of root hairs and freedom from the normal (?) fungus associations on them, account for their comparative inefficiency as absorbing organs. This apparently explains the difficulty in transplanting plants having many large roots but no fibrous ones as compared with those having at least a few small ones.

SUMMARY

The most distinctive features observed in the strawberry (var. Progressive) are:

1. There are two types of roots; large adventive ones, and fibrous branch roots, which show the following perfectly distinct and characteristic morphological differences:

a. The tissues of the adventive roots differentiate very tardily; those of the fibrous branch roots differentiate early.

b. The vascular bundles of the former are placed far apart; those of the latter are very closely approximated.

c. The former have a large central medulla; in the latter the central medulla is entirely wanting.

d. The former very rarely show secondary thickening; in the latter secondary thickening occurs abundantly.

e. The steles of the adventive roots are tetrarch, pentarch, hexarch, or polyarch; those of the branch roots are diarch, triarch, or tetrarch only.

f. In the former the pericycle is many layers in thickness; in the latter it is of a single layer.

g. In the former the endodermis is unmodified; in the latter the endodermis is greatly thickened.

h. The adventive roots are without definite fungus-bearing regions, while in the branch roots a mycorrhizal (?) fungus is commonly present in a definite region of the cortex.

2. There is a many-layered pericycle in the crown and in the adventive roots.

3. Numerous oblique and transverse conducting strands are present in the mature crown.

4. The tracheids are provided with highly peculiar pores in their lateral walls, which greatly increase the possibility of tangential movement of material within a single xylem strand.

The physiological importance of these features needs to be further examined experimentally, and the anatomical characters, particularly in regard to their occurrence in related genera, need further study.

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COMPARATIVE STUDIES OF WINTER HARDINESS IN WHEAT [1]

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THE WINTER-HARDINESS PROBLEM IN WHEAT

Winterkilling is practically the only factor which limits the growing of winter wheat in northern regions, especially in the north-central part of the United States. The losses due to winterkilling in the United States, the methods of reducing winter injury, and the hardiness of certain varieties have been shown previously (*10*, *11*, *26*, *28*).[3]

Several difficulties are encountered in determining the hardiness of wheats by field methods. Winterkilling of wheat plants in the field is very irregular, and complete killing or complete survival of the plants frequently occurs. Repeated field tests are necessary to measure hardiness in varieties. Poorly adapted varieties often show a low winter survival even though they are known to be resistant to cold (*14*). Frequently it is not low temperatures, but drought, soil blowing, soil heaving, and smothering by ice which are responsible for the killing of winter-wheat plants. The low correlation between the survival of winter-wheat hybrid strains and that of selections from them was shown by Vassar.[4] In the writer's experiments no significant correlations were obtained between the survival of hybrid strains and selections from them. A laboratory method for measuring winter hardiness, which would be more accurate than field observation, therefore, is greatly needed.

The complexity of the problem and the differences between varieties have made positive conclusions regarding the nature of hardiness rather difficult. Many characters possessed by hardy wheat varieties also are found in nonhardy varieties or they do not occur in rye, which is hardier than wheat. The hard red winter wheats are the hardiest group known. These wheats are characterized by narrow, dark-green, prostrate leaves in the seedling and rosette stages. The narrow leaves seem to be fairly well associated with hardiness in certain American winter wheats but not necessarily in all groups of wheat. The dark color and prostrate position of juvenile leaves also are not definitely related to hardiness. Any relationship between hardiness,

[1] Received for publication Dec. 2, 1926; issued November, 1927. A thesis submitted to the faculty of the Graduate School, University of Minnesota, in partial fulfillment of the requirements for the degree of doctor of philosophy, June, 1926. Most of the investigations reported herein were conducted at the Minnesota Agricultural Experiment Station, University Farm, St. Paul, Minn., during the years 1923-24 and 1925-26.

[2] The writer wishes to express his thanks to Dr. R. A. Gortner, professor of agricultural biochemistry at the University of Minnesota, for his help in outlining these experiments and in providing facilities for carrying them out; to Dr. H. K. Hayes, professor of plant breeding, and other members of the staff of the Minnesota Agricultural Experiment Station, for materials and equipment furnished and suggestions given during the course of these investigations; and to Dr. Robert Newton, professor of field husbandry at the University of Alberta, for supplying the writer with some of his experimental results on winter hardiness of wheat in advance of their publication by him.

[3] Reference is made by number (italic) to "Literature cited," p. 532.

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all anatomical characters, and nearly all physiological characters probably could be disproved by the proper selection of a limited number of cereal varieties.

The recent work of Newton (*34*, *35*, *36*) shows, however, some important physiological characteristics of several of the hardy wheat varieties, which might be used in measuring the hardiness of varieties and hybrids.

REVIEW OF LITERATURE ON WINTER HARDINESS IN PLANTS

CAUSES OF WINTERKILLING

In spite of abundant data to the contrary the belief of the old Greek philosophers and of Duhamel and Buffon as late as 1740 (*13*) that frost injury in plants is due to rupture caused by growing ice crystals within the cells has not yet been entirely dissipated. Göppert (*15*), Sachs (*44*), Müller-Thurgau (*32*, *33*), Cavallero (reported by Abbe (*1*)), Molisch (*30*), and Wiegand (*56*) observed that ice formation takes place mostly in intercellular spaces and that the rupture of cells by freezing rarely occurs. Müller-Thurgau (*32*, *33*) reported thta ice formation takes place within the cell only on rapid cooling, and Cavallero (*1*) stated that, even though ice is formed within the cells, the protoplasm never freezes. Molisch (*31*), Müller-Thurgau (*32*, *33*), and Wiegand (*56*) stated that frost injury was due to the withdrawal of water from the cells by freezing.

Other workers, including Cavallero (*1*) and Schindler (*51*), believed that frost resistance was due to chemical peculiarities of the cell protoplasm. Frost injury was believed by Gorke (*16*), Lidforss (*24*), Schaffnit (*48*), and Schander and Schaffnit (*50*) to be due to the precipitation of cell proteins, through salting out by the increased concentration of the cell sap after water had been withdrawn in freezing. Voigtländer (*55*) and Chandler (*9*) disagreed with this theory, the latter finding that plants grown with an abundance of nutrient salts are hardier and their protoplasm less easily precipitated than is the case with plants grown with a poorer supply of nutrients. The higher sap concentration of the better nourished plants, however, probably tends to increase imbibition; in which case Chandler's objection is not necessarily valid.

Mez (*29*) believed death to be due to cooling a plant below its specific minimum and held that the prevention of undercooling during freezing sometimes keeps the temperature of a plant above its specific minimum. Apelt (*4*) found that undercooling will not continue long, while Voigtländer (*55*) stated that undercooling rarely occurs in nature and is not related to the killing point or the death of the plant. Sachs (*44*) believed death from cold resulted from several factors, including shrinkage and disorganization of the cell following ice formation, ice expansion injury, and destruction by thawing after freezing. Detmer (*12*) stated that plant tissues were killed during freezing, because a change in the color of the chlorophyll could be observed.

The recent view appears to be that frost killing consists in the withdrawal of water from the cells to form ice in the intercellular spaces, followed by the precipitation of the protoplasm, due chiefly to the increased concentration of the cell sap.

THE NATURE OF COLD RESISTANCE

The factors which frequently have been reported concerned with cold resistance include low moisture content, increased sugar content[5] (*2, 3, 4, 18, 24, 36, 48*), increased sap concentration, increased colloidal material, and a long vegetative period (*8, 51*), as well as certain morphological characters of wheat, such as narrow or prostrate leaves (*8, 45*) and large cells (*5*).

The relation of low moisture content to hardiness in wheat was shown by Sinz (*53*) and Newton (*35*). The same relationship was shown in apple twigs by Shutt (*52*) and by Beach and Allen (*7*), in peach twigs by Johnston (*21*), in plum twigs by Strausbaugh (*54*), and it has been indicated for vegetable plants by Rosa (*43*).

Although Maximow (*27*) and Chandler (*9*) found that increasing the sap density of plants increased the resistance to freezing more than could be accounted for by the increased osmotic pressure, Salmon and Fleming (*46*), Pantanelli (*40*), and Baroulina (*5*) found no definite relation between osmotic concentration of the sap and the cold resistance of cereals. The failure to find a relation probably was due to the selection of varieties, the attempt to compare different crops, or the time of collecting samples. The method of extraction influences the freezing point of the sap, and it may differ considerably from the freezing point of the tissues, as shown by Newton and others (*38*) and by Wright (*58*).

Imbibitional pressure due to hydrophilic colloids, as measured by the quantity of juice expressed from wheat leaves, was shown by Newton (*34, 35*) to be closely related to hardiness, the more hardy varieties yielding the lesser quantity of juice. The relation of total solids and bound water to hardiness was pointed out by Newton and Gortner (*37*).

Rosa (*43*) reported pentosan content to be related to hardiness in vegetable plants, but Newton[6] did not find this relationship in wheat. Recent experiments by McGinty (*25*) indicate that the methods used by Rosa would not actually measure pentosan content.

Zacharowa (*59*) reported the most cold-resistant tissues to be the most alkaline and to contain a lower salt concentration and a higher protein content. Kolkunov (*23*) reported that xerophytic wheat varieties resist injury from low temperatures.

Newton (*35*) suggested that, because of the better retention of sugars, hardy cereals may have a lower rate of respiration than nonhardy ones, which has been shown by Govorov (*18*) to be the case.

LETHAL TEMPERATURES FOR CEREAL PLANTS

Schaffnit (*49*) found that wheat seedlings were injured very little at $-5°$ to $-10°$ C. but were considerably injured at $-15°$ to $-20°$ C. Both the degree and the duration of cold affect the extent of killing. Klages (*22*) exposed wheat plants to cold for short periods and obtained results similar to those of Schaffnit. Rye seedlings three or four days old were found by Zacharowa (*59*) to be killed at $-7.8°$ but not at $-2.9°$ C. At $-5.75°$ and $-3.9°$ C. the seedlings were killed except at the root tips. Buhlert (*8*) froze the juices of spring

[5] NEWTON, R. THE NATURE OF WINTER HARDINESS IN CROP PLANTS. Progress Reports to the Honorary Advisory Council for Scientific and Industrial Research (Canada). (Typewritten reports, May, 1924, and May, 1925, furnished the writer by courtesy of Dr. Robert Newton.)

[6] NEWTON, R. Op. cit.

barley, winter barley, and winter rye and found at −7.0°, −12°, and −15° C., respectively, a precipitate consisting of protein with traces of lime and phosphoric acid.

PROBLEMS INVESTIGATED

The chief object of the investigations undertaken by the writer was to determine the most accurate and feasible methods for measuring winter hardiness in wheat varieties and hybrids. Four general problems were studied: (1) Laboratory investigations of factors for hardiness; (2) determination of lethal temperatures for wheat; (3) hardiness of plant parts; and (4) hardiness in wheat hybrids. Other objects of the investigations were to determine further the processes of hardening, the causes of winterkilling, and the inheritance of winter hardiness in wheat plants.

LABORATORY INVESTIGATIONS OF FACTORS FOR HARDINESS

The factors which were investigated in the laboratory studies included the moisture content and imbibitional pressure of the leaves, total solids, freezing-point depression, and bound water in the expressed juice or sap, the seasonal changes in these factors, and the rate of respiration of wheat varieties at low temperatures.

MATERIALS AND METHODS USED

Four varieties of wheat—Minhardi, Kanred, White Winter, and Marquis—were used in these investigations. Minhardi has been shown (*11*) to be the hardiest winter-wheat variety grown in the United States. Kanred, an important hard red winter variety, is less hardy than Minhardi. White Winter was selected because it appeared to be among the least hardy of the winter wheats. Marquis, a spring wheat, was used for comparison with the winter wheats because of its relative hardiness among the spring wheats. Swedish (Minnesota No. 2) rye was included in the experiments because it was known to be considerably more hardy than Minhardi wheat and to determine whether the same physiological factors for hardiness occur in wheat and rye.

The moisture content of the leaves was determined from samples of approximately 10 grams, weighed before and after drying 24 hours or more in an oven at 100° C. The imbibitional pressure of the leaves was determined by measuring the quantity of juice or sap per gram of green tissue expressed by a hydraulic press at pressures of 50, 150, 250, and 350 kgm. per square centimeter. Samples, usually consisting of 25 to 75 gm. of tissue, were wrapped in pieces of boiled unbleached muslin 5 inches square. They were then placed in a steel press bowl and the hydraulic pressure applied to a piston in the bowl.

The percentage of total solids in the sap was determined by means of the Abbe refractometer, the sugar scale being used as a basis for estimating solids, as suggested by Gortner and Hoffman (*17*). Freezing-point depressions were determined with a Beckmann apparatus, a salt-and-ice bath being used. The percentage of bound water in the sap was ascertained by the method of Newton and Gortner (*37*), by determining the freezing-point depression of the

sap before and after adding pure sucrose in a quantity equivalent to a molecular concentration of sucrose in the water of the sap. A quantity of sap containing 10 gm. of water, as determined by the percentage of total solids, was weighed out, 3.422 gm. of sucrose added, and the freezing-point depression then determined.

For example, a sample of sap showed a freezing-point depression of 1.51° C. With a quantity of added sucrose necessary to give a molar concentration, it had a freezing-point depression of 3.86° C., which, minus 1.51°, equals 2.35°, the excess freezing-point depression. Of this excess, 2.085° was due to the sucrose, which in solution forms sucrose hexahydrate. Then 2.35° minus 2.085° equals 0.265°, which is the excess freezing point due to water being bound by colloids in the sap so that it can not dissolve the sucrose. Dividing 0.265 by 2.35 and multiplying the result by 89.2 gives 10.1 per cent of bound water. The figure 89.2 shows the quantity of free water in 100 c. c. of a molar solution of sucrose in which 6 molecules or 10.8 per cent of the water is taken up to form sucrose hexahydrate.

The lethal temperatures for wheat plants were determined by subjecting the plants growing in pots to freezing, usually for 24 hours at known temperatures, and then returning the pots to the greenhouse and noting the percentage of plants which recovered. The rate of respiration was measured by drawing a stream of air free from carbon dioxide through a closed chamber covering the wheat leaves, which chamber was sealed off from the laboratory air and from the soil in which the pots were growing by a half-and-half mixture of vaseline and liquid paraffin. The air was caused to flow through the chamber by equal suction and pressure at the rate of 18 liters per hour. Five volumes of air passed through the chambers during each half hour of aspiration, as the capacity of the chambers was 1.8 liters. The chambers were aspirated for half-hour periods whenever a sufficient quantity of measurable carbon dioxide had accumulated. The carbon dioxide given off by the plants was collected in bead-absorption towers containing a standard solution of barium hydroxide, and the excess of alkali was titrated with HCl, using phenolphthalein as an indicator. The acid solution was of such strength, N 0.04545, that each cubic centimeter was equivalent to 1 mgm. of CO_2. The temperatures during respiration were kept constant to within about 0.1° C. by placing the potted plants in the chambers of an ice-cream cabinet which were electrically heated by means of lamps, to keep the temperatures in the chambers above that of the surrounding bath. The temperatures were controlled by thermoregulators. (Fig. 1.)

The wheat used in these investigations of the tissue-fluid properties was sown in 8-foot rows on fall-plowed land. The sowing was done by hand, September 11 and 12, in 1923, at the rate of 5 gm. per row or 1 bushel per acre. In 1925 it was sown with a hand planter on August 27, at the rate of 5.5 gm. per row. The rye was sown September 21 in 1923, but in 1925 it was sown on the same date as the wheat. Duplicate plots were sown consisting of 25 rows of each variety in 1923 and 50 rows in 1925. In 1923, delayed rains caused a slow growth of wheat, and the plants had reached only the three-leaf stage when the first samples were gathered October 19. Growth continued, however, until the latter part of November. In 1925,

much better early growth occurred, but there was not much increase in dry matter after October 15. Heavy wrapping paper was placed each year over the wheat plants after they had stopped growing to keep the snow from them. This facilitated the gathering of samples when the ground was covered with snow.

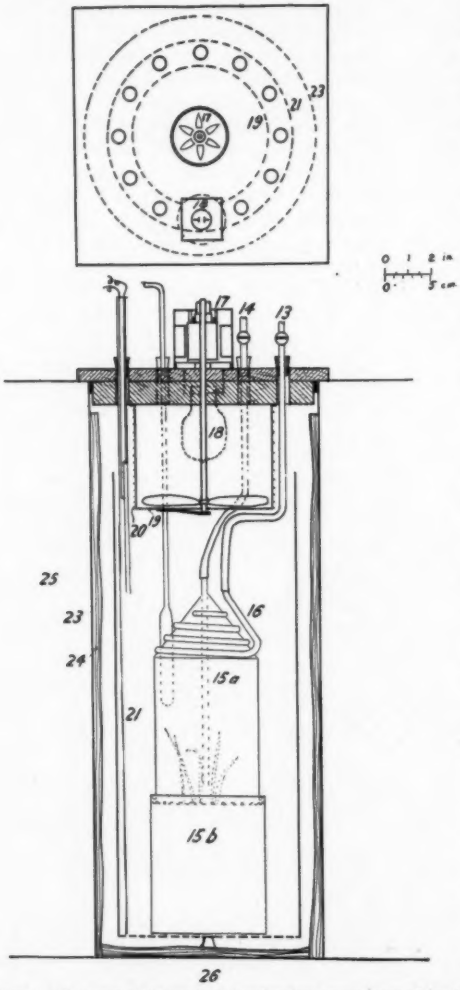

FIG. 1.—Diagram of the constant-temperature apparatus used for the respiration experiments: 13, Tube for ingoing air; 14, tube for outgoing air; 15a, sheet-metal respiration chamber; 15b, earthenware jar containing growing plants with the soil surface sealed off; 16, coil of copper tubing for cooling air before it enters respiration chamber; 17, fan motor; 18, socket and light for supplying heat; 19, support for fan shaft bearing and fan guard; 20, cylindrical wire screen surrounding the fan blade; 21, sheet-metal cylinder; 23, wall of cell of freezing cabinet; 24, asbestos lining for insulating the cell; 25, fluid of the freezing cabinet; 26, cork insulation of freezing cabinet

Duplicate samples of each variety were collected from adjoining rows at intervals of three or four days during the autumn and at greater intervals after December 15. Nearly all of the samples were collected between 10 a. m. and noon. The plants were cut off at the surface of the soil with shears and placed in screw-top bottles. They were then taken to the laboratory, and about 10 gm. of tissue was removed from the top of each bottle for moisture determinations. The remainder of each sample was weighed and taken immediately to the "hardening" room, located in the dairy building, where it was maintained in a frozen condition. Samples which were frozen when collected were not permitted to thaw. The hardening room varied in temperature usually between $-12°$ C. (10° F.) and $-23°$ C. ($-9°$ F.) but dropped to the latter figure nightly. After they had been left 24 hours or more in the hardening room, the samples were returned to the laboratory and pressed. In 1923, one of the duplicate samples of each variety was pressed soon after it was collected and

without preliminary freezing. In 1925, both samples of each variety were frozen.

The daily maximum and minimum temperatures recorded at St. Paul, Minn., during the fall and winter months from October to February, inclusive, in 1923–24 and 1925–26, are shown in Table 1. These data are given because of their relation to the hardening-off process in the wheat plants under field conditions, this relation being reflected in the data obtained on the different sampling dates as hereafter given. In 1923 the first freeze occurred on October 19, the date of the first collection of winter wheat from the field, but the wheat plants apparently had not been frozen. In 1925 the first collection was made on October 12, after light frosts had occurred, but while the plants were still in an actively growing condition.

TABLE 1.—*Daily maximum and minimum temperatures at University Farm, St. Paul, Minn., during stated fall and winter months of 1923–24 and 1925–26*

Day	Temperature (° F.)																			
	1923–24										1925–26									
	Oct.		Nov.		Dec.		Jan.		Feb.		Oct.		Nov.		Dec.		Jan.		Feb.	
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum
1	66	47	52	23	43	19	−1	−19	32	28	57	43	50	27	35	12	36	20	21	9
2	72	42	45	40	35	11	10	−3	41	29	63	48	50	28	39	32	38	21	25	10
3	71	44	52	35	43	23	−2	−19	35	26	59	44	48	33	36	32	30	20	27	13
4	61	42	48	28	44	27	−9	−22	26	9	52	42	53	29	33	12	37	30	20	11
5	59	36	52	30	44	16	−6	−27	11	3	56	37	40	24	13	7	35	24	37	10
6	62	38	58	32	40	26	36	−6	12	3	51	31	31	19	20	11	26	11	37	24
7	62	44	43	26	45	26	31	16	20	3	41	38	25	12	32	14	18	4	41	16
8	59	41	41	22	45	29	32	22	26	1	42	37	29	8	39	25	22	6	35	29
9	75	48	58	31	33	21	30	20	28	18	39	30	49	23	28	21	22	5	32	19
10	77	52	59	37	35	20	23	15	37	12	45	26	52	30	33	20	33	22	24	8
11	71	54	60	31	48	21	15	6	33	24	59	41	56	30	33	30	30	−5	31	11
12	58	43	49	41	42	17	6	−8	32	17	56	36	52	27	34	13	23	−7	33	27
13	53	39	50	45	25	10	5	−11	39	27	58	43	47	30	21	11	22	2	33	26
14	52	34	48	43	41	14	13	−14	37	18	52	42	32	30	21	8	36	16	29	12
15	65	35	45	38	47	30	24	8	16	3	58	37	32	27	25	14	28	8	17	6
16	66	53	38	30	46	33	8	−16	26	12	44	32	30	22	19	10	36	25	41	5
17	69	53	55	28	52	32	6	−16	25	17	44	27	49	22	12	5	37	21	32	15
18	53	37	47	33	54	28	10	−1	22	14	33	29	50	34	22	2	38	22	27	9
19	44	30	50	28	49	34	2	−9	24	16	38	30	45	32	25	17	26	4	25	11
20	47	30	51	37	43	38	−6	−20	23	10	35	30	61	34	24	15	12	−5	21	12
21	52	27	38	20	44	35	14	−21	16	2	47	26	45	26	15	9	4	−13	21	11
22	58	31	37	16	36	28	34	14	21	−2	32	27	26	16	16	11	5	−17	23	10
23	60	34	36	28	33	23	17	1	23	5	37	31	36	16	20	9	24	−5	43	19
24	55	36	41	28	33	23	29	0	27	6	41	32	43	27	17	8	2	−9	36	30
25	42	37	50	33	35	25	0	−15	36	11	42	30	37	23	10	−3	17	−6	37	31
26	44	39	33	26	42	26	0	−11	43	16	37	25	38	16	−2	−13	23	−1	31	16
27	42	34	31	19	35	13	22	−8	44	19	26	20	18	5	5	−10	13	−13	34	12
28	43	32	41	19	26	9	36	17	39	26	24	17	21	5	6	−6	8	−20	48	31
29	34	22	46	27	20	8	40	24	33	28	23	13	23	19	14	−8	34	8	----	----
30	32	19	46	23	8	0	37	23	----	----	27	8	31	----	16	1	36	25	----	----
31	44	24	----	----	11	0	35	28	----	----	42	24	----	----	32	7	31	23	----	----
Mean	56.4	38.0	46.7	29.9	38.0	21.5	15.8	−1.7	28.5	13.8	43.9	31.5	40.0	23.2	22.4	10.2	25.2	7.0	30.8	15.8

The temperatures in 1923 continued rather mild, and the wheat continued to grow until well into November and resumed growth again during the few days following December 15. During the night of December 26 the temperature dropped abruptly and continued low until January 6, 1924. During this period the growth

of wheat stopped and the plants remained frozen and were covered with snow. The sudden change of temperature caught the plants in an unhardened condition, but in spite of this they survived the minimum temperature of −33° C. (−27° F.) of January 5 with little or no injury. This was due to the 3-inch covering of snow. Thermograph records of the air temperature and the soil temperature just below the surface on a plot a few rods from the wheat plants were kept by A. C. Arny, and some of the results were shown to the writer. During the first two weeks of January, when the air temperature dropped to a minimum of −33° C. (−27° F.) and rose to a maximum of about +2° C. (+36° F.), the soil temperature remained practically constant at −2° C. (+28° F.), which is not fatal to any variety of winter wheat. After January 1 the wheat remained covered with some snow until spring.

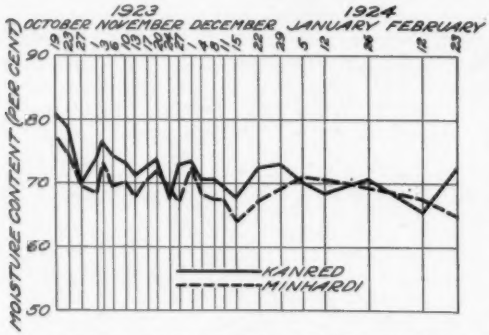

FIG. 2.—Moisture content of Kanred and Minhardi wheat leaves collected at intervals during the autumn and winter of 1923–24, University Farm, St. Paul, Minn.

In the autumn of 1925 the wheat apparently continued growth until the latter part of October and remained vegetatively active until the last week in November. The temperatures during December and January were relatively low, and there was some snow on the ground continuously. The wheat plants remained frozen nearly all of the time from the latter part of November until early in March, although the plants collected on January 16 had thawed somewhat.

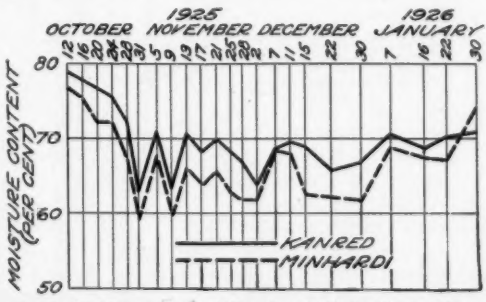

FIG. 3.—Moisture content of Kanred and Minhardi wheat leaves collected at intervals during the autumn and winter of 1925–26, University Farm, St. Paul, Minn.

The official temperatures recorded were taken with instruments placed in a shaded inclosure, while in the field on clear days the sunshine frequently caused the plants to thaw and take up water from the soil, even when the maximum temperature recorded in the instrument shelter was below freezing. The wheat plants thus were able to recover water to replace that lost by freezing and evaporation, apparently changing their imbibitional power during periods in which, on the basis of recorded temperatures, they would be expected to remain frozen and unchanged.

The winter of 1923–24 might be considered an abnormal one, on account of the long-continued growing weather and the high temperatures in December, followed by very low temperatures in early January. The absence of low temperatures except when the ground was covered with snow permitted almost complete survival of winter wheat. The season of 1925–26, on the other hand, was more nearly normal, as temperatures tended to decrease gradually until the soil was frozen and covered with snow at the end of November. The conditions prevailing during that season and their effects on winter wheat were about such as might be expected in most seasons in sections having a climate similar to that of St. Paul, Minn. Rather heavy winterkilling occurred in the winter-wheat nurseries.

RESULTS OBTAINED

The data obtained from the laboratory investigations of factors for winter hardiness in wheat are shown in detail in the following pages. In the discussion following the presentation of these and other experimental results, the relations of the various factors concerned with hardiness and hardening are considered.

Moisture Content

Data showing the average moisture content of duplicate samples of wheat leaves collected during the two seasons are given in Table 2 and are shown graphically in Figures 2 and 3. The moisture content of the leaves of the different varieties was about 80 per cent during early October, when the plants were actively growing. With the approach of cold weather the moisture content dropped to about 70 per cent. Most of this decrease resulted from the wilting of the tissues following freezing. After each freeze some of the lost moisture was recovered. This explains the wide fluctuation in moisture content shown in Table 2. Dead tissues were not entirely eliminated from the moisture samples. The separation of the dead tissue is very difficult when considerable quantities of partly wilted tissue, are present. It is not known just how little moisture the tissues could contain without being wilted in appearance, but, in general, all samples collected in late autumn or winter which showed less than 68 to 70 per cent moisture were at least partly wilted. The hardy varieties could contain less moisture than the less hardy varieties without being wilted.

From the data of Table 2 it is evident that the Kanred variety had a higher moisture content than the Minhardi on all but five dates. These exceptions followed severe freezes in which Kanred was more injured than Minhardi. The difference in moisture content may well be considered a measure of hardiness in which the hardier variety has the lower moisture content. White Winter wheat, when not too badly wilted, showed a higher moisture content than either Kanred or Minhardi. Rye tends to have a high moisture content, even though it is extremely hardy. This, however, does not disprove the relationship in wheat, which belongs to another genus.

TABLE 2.—*Moisture content of the plants of four varieties of wheat and one variety of rye collected on stated days during the seasons of 1923–24 and 1925–26, University Farm, St. Paul, Minn.*

Date collected	Moisture content (per cent)				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
Season of 1923–24					
October:					
19	77.8	81.1			
23	74.7	78.9			
27	69.6	69.8			
November:					
1	68.6	73.7			
3	73.2	76.9	76.0		76.3
6	69.7	74.6			
10	70.2	73.4	72.6		75.2
13	67.8	71.3			
17	70.6	72.9	73.6		74.2
20	71.9	73.9			
24	69.1	67.8	73.4		69.6
27	66.9	72.8			
December:					
1	72.5	73.6	73.2		72.0
4	68.7	70.9			
8	67.5	70.9	69.1		
11	67.4	69.7			
15	64.2	67.9	57.3		
22	67.2	72.5	67.5		
29	69.2	73.1	72.7		
January:					
5	71.1	70.3	66.7		
12	70.7	68.5	68.6		
26	69.4	70.8	75.2		
February:					
12	67.7	65.7	69.2		
23	65.0	72.4	64.5		
March:					
8	56.7	63.7	64.3		
27	60.9	57.0			
May:					
17	75.1	78.7			
Average [a]	68.677 ±0.512	71.219 ±0.590	69.593 ±0.847		
Season of 1925–26					
October:					
12	76.8	78.7	81.2	81.8	80.2
16	75.4	77.5	80.5	80.9	78.4
20	72.2	76.7	78.4	78.5	77.4
24	72.1	75.5	77.2	77.0	74.8
28	67.4	72.1	72.9	69.5	72.1
31	59.3	62.9	64.0	65.4	64.9
November:					
5	67.8	70.8	71.0	71.8	72.7
9	59.6	63.2	64.5	57.6	65.5
13	66.0	70.8	70.5	66.1	71.1
17	63.7	68.1	68.1	60.4	68.4
21	65.6	69.9	69.8	68.4	70.0
25	62.7	68.1	65.5	60.7	71.1
28	61.7	66.9	66.2	62.4	70.7
December:					
2	61.9	63.8	62.0	59.2	67.7
7	68.5	68.7	66.0	64.7	71.9
11	68.0	69.7	68.6	69.1	72.6
15	62.6	69.0	65.1	61.2	73.1
22	62.3	65.8	62.3	62.2	71.3
30	61.8	66.6	57.5	58.7	70.4
January:					
7	68.8	70.6			72.5
16	67.4	68.8	65.1	66.3	69.1
22	67.0	70.2			68.6
30	74.0	70.9	76.2	60.2	67.9
May:					
4	73.5	77.7			79.2
Average [b]	66.635 ±0.682	69.796 ±0.576	69.171 ±0.933	66.767 ±1.071	71.409 ±0.518

[a] Not including samples collected Oct. 19.

[b] Not including samples collected May 4.

The data on moisture content for 1925–26 are more complete than those for 1923–24. In 1925, on October 12, 16, 20, and 24, the moisture contents of Marquis and White Winter were distinctly higher than those of the hardier wheats, Minhardi and Kanred. Rye again showed a moisture content similar to that of the nonhardy varieties. During the autumn of 1925 the moisture content of all varieties decreased until about December 2, when a certain recovery occurred. The moisture contents of White Winter and Marquis leaves were relatively low after October 28. During this season the moisture contents again showed large fluctuations due to freezing and thawing, with consequent wilting and recovery. The higher moisture content of Kanred, as compared with Minhardi, was maintained throughout the season except on January 30. It is evident from this and from the behavior of White Winter and Marquis wheats that varieties somewhat similar in hardiness tend to fluctuate together, while widely different varieties are dissimilar because they are affected in widely different degrees by freezes or by their ability to recover. True comparisons in moisture content can be obtained only when the plants are not affected by freezing and wilting. On October 12, 16, and 20, the four wheat varieties had moisture contents in the inverse order of their hardiness. After that time this relationship did not exist. The lowest moisture contents of the nonhardy varieties were less than the lowest moisture contents determined for the hardy varieties, because the former contained more wilted and dried leaves.

Duplicate samples of 10 varieties of winter wheat and one variety of rye were collected for moisture determinations, from nursery rows, on May 31, 1924. The plants at that time had almost reached the jointing stage. The moisture-content data are given in Table 3 with the wheat varieties arranged in the order of their hardiness. Rye, although much hardier, had a moisture content higher than any of the wheats. The four hardiest wheat varieties—Minhardi, Buffum No. 17, Minturki, and Odessa—had moisture contents lower than the less hardy varieties, although the order of the moisture contents and the relative hardiness of these varieties among themselves was inverse to that of all varieties as a group. The remaining varieties with somewhat higher moisture contents also failed to show a definite relation between hardiness and moisture content. Differences in earliness and consequent stages of growth, however, may have affected the results.

On November 16, 1925, triplicate samples of five varieties of wheat were collected from the border rows of the varietal plots at University Farm. Collections were not made early in October, because sufficient growth had not been attained, and the samples collected November 9 were too badly wilted to show reliable results. The moisture contents of the five varieties, listed in their order of hardiness, are shown in Table 3. While again no definite relation between hardiness and moisture content were shown, Red Rock, the least hardy variety, contained decidedly more moisture than any other variety, and Kanred, the second least hardy variety, showed the second highest moisture content.

TABLE 3.—*Average moisture content of samples of the plants of varieties of winter wheat and rye collected at University Farm, St. Paul, Minn., and listed in the order of average hardiness*

Duplicate samples collected May 31, 1924				Triplicate samples collected Nov. 16, 1925	
Variety	Moisture content	Variety	Moisture content	Variety	Moisture content
	Per cent		*Per cent*		*Per cent*
Minhardi	74.9	Kanred	76.1	Minhardi	79.0
Buffum	73.4	Kharkof	76.7	Minturki	77.9
Minturki	73.3	Blackhull	76.4	Turkey	77.2
Odessa	72.7	Nebraska No. 28	75.5	Kanred	79.2
Padul	76.9	Minnesota No. 2 rye	81.2	Red Rock	82.1
Turkey	75.7				

An experiment to determine the rate at which wheat plants dry out at low temperatures was conducted by the use of artificial refrigeration. At the beginning of the experiment, samples taken from pots of Kanred and Minhardi wheat growing in the greenhouse contained 75.5 per cent and 73.4 per cent of moisture, respectively. Other pots containing plants of these varieties were then exposed successively to the following temperatures during the periods named: 1° C. for 24 hours, −8° C. for 36 hours, and −12° C. for 8 hours. At the end of this period Kanred contained 73.3 per cent and Minhardi 72.8 per cent of moisture. The remaining plants were then maintained at temperatures between −2° and −4° C. for one week. Under these conditions the plants were thawed, but the soil remained frozen. At the end of the week the moisture content of the Kanred samples was reduced to 36.6 per cent and that of the Minhardi samples to 41.6 per cent. These latter samples of the two varieties consisted of the remaining plants from the same pots from which the previous samples had been taken. Duplicate pots of Kanred were returned to the greenhouse after the exposure at −12° C.

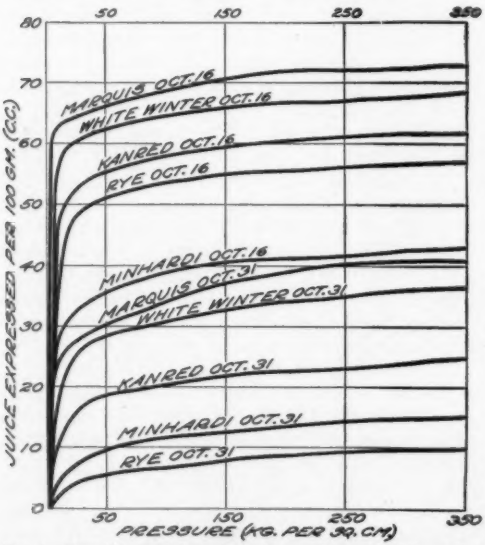

FIG. 4.—Quantities of juice expressed per 100 grams of tissue from frozen leaves of four varieties of wheat and one variety of rye at pressures of 50, 150, 250, and 350 kgm. per square centimeter, on October 16 and October 31, 1925, University Farm, St. Paul, Minn.

All of the Kanred plants and half of the Minhardi plants had been killed by this exposure. The subsequent rapid drying was largely

in dead tissue. Under usual freezing conditions most of the leaves are killed. The experiment just described merely demonstrates that even at freezing temperatures drying of wheat leaves may take place rapidly.

It does not seem possible to differentiate between varieties which are rather similar in hardiness by merely determining their moisture contents. Under more favorable conditions for obtaining representative samples, true differences in hardiness might be shown, especially if several samples were taken and the results averaged.

Quantity of Juice Expressed from Leaves

The imbibition pressure of wheat leaves, as measured by the volume of juice or sap expressed from the leaves by given pressures, was found by Newton (*34, 35, 36*) to be closely related to hardiness. This test was applied in these studies during both seasons, and in 1923 on both unfrozen leaves and leaves frozen in the hardening room before pressing. In 1925 all samples were frozen before pressing.

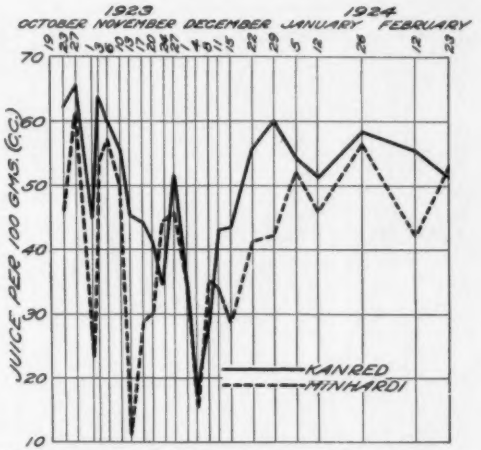

Fig. 5.—Quantities of juice expressed, at a pressure of 339 atmospheres, per 100 grams of frozen leaves of Kanred and Minhard wheats collected at intervals during the autumn and winter of 1923-24, University Farm, St. Paul, Minn.

Although measurements of expressed sap were recorded for pressures of 50, 150, 250, and 350 kgm. per square centimeter, only the quantities expressed at 350 kilograms are presented. All of these measurements, as shown by the curves in Figure 4, showed approximately the same relative values. At 50 kgm., frozen samples of Kanred and Minturki yielded, respectively, 80.6 and 79.5 per cent; at 150 kgm., 91.5 and 92 per cent; and at 250 kgm., 95.4 and 96.5 per cent of the quantity of sap expressed at 350 kgm. It was found by experiment that about 1 c. c. of sap was absorbed on the cloth, bowl, and plunger in pressing; and as the size of sample pressed varied considerably, the data for sap expressed per 100 gm. of material are corrected by adding 1 c. c. to the actual quantity of sap expressed. The pressure readings taken were in kilograms per square centimeter. A pressure of 350 kgm. per square centimeter is equal to 338.7 atmospheres, and approximately the latter measure (339 atmospheres) is used in the tables and textual discussion.

The data for the quantity of juice expressed per 100 gm. of fresh tissue at a pressure of 339 atmospheres, together with the percentage of total moisture which was expressed, are given in Tables 4 and 5 and are shown graphically in Figures 5 and 6.

For unfrozen leaves, the quantity of juice expressed per 100 gm. of unfrozen tissue decreased during the season from 25.4 c. c. for Minhardi and 29.1 c. c. for Kanred on October 19, to 5.2 c. c. for Minhardi and 5.3 c. c. for Kanred on December 8. After the latter date the quantity increased somewhat. A similar relation was shown in the percentages of total moisture expressed, indicating that the decreased quantity of expressed juice was not entirely a measure of the decreased moisture content. More juice was expressed from rye and White Winter wheat than from Kanred and Minhardi wheats, because of their higher moisture content.

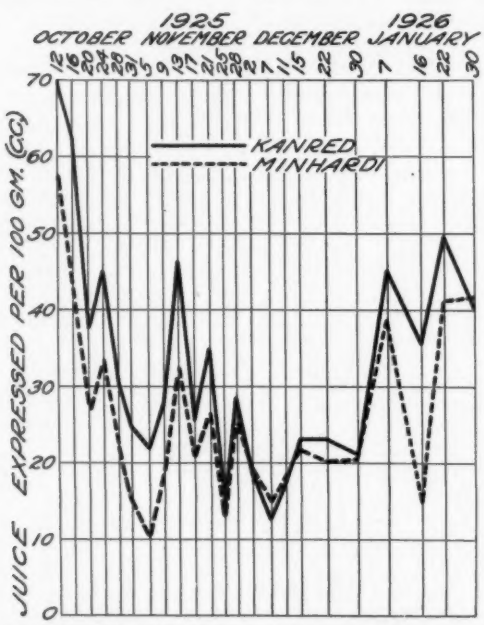

FIG. 6.—Quantities of juice expressed, at a pressure of 339 atmospheres, per 100 grams of frozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1925-26, University Farm, St. Paul, Minn.

The data in Table 4 show that considerably more juice is expressed from the frozen than from the unfrozen tissues. This is due to the increased permeability of the cell membranes and the partial precipitation of the protoplasm as a result of freezing. More juice was expressed from Kanred wheat than from Minhardi and more from White Winter than from Kanred. The quantity of juice expressed from these varieties of wheat previous to the November 20 samplings was always in the inverse order of their hardiness. After that date there were some irregularities, but usually the less hardy varieties yielded more juice than Minhardi. The differences between Kanred and Minhardi in the quantity of juice expressed varied considerably, depending not only upon the moisture content but also upon the effects of freezing upon the tissues. After November 20 the differences were too irregular or too small to be of value in distinguishing between hardy and nonhardy wheats, and after December 4 the quantity of juice obtained from both varieties was too large to be used as a basis for measuring hardiness.

More juice was expressed from rye than from Minhardi wheat until December 1, the date of the last collection of rye. There was little indication from the results obtained in 1923 that rye possessed greater ability to hold water against freezing and pressure than the hardy winter wheats. This may have been partly because the rye was sown nine days later than the wheat. Approximately the same

relationships were shown in percentage of total moisture expressed as in the quantities of juice expressed. The percentage expressed from White Winter wheat was more than 70 per cent except on two occasions, while Minhardi yielded less than 70 per cent of its juice most of the time, except at the beginning and end of the season.

TABLE 4.—*Quantity of juice expressed, at 339 atmospheres pressure, from frozen and unfrozen plants of three varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1923–24, University Farm, St. Paul, Minn.*

Condition and date collected	Juice per 100 gm. of fresh tissue (c. c.)				Percentage of total moisture expressed			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
UNFROZEN PLANTS								
October:								
19	25.4	29.1			32.6	35.9		
23	15.4	14.1			20.6	17.8		
27	16.8	20.3			24.1	28.9		
November:								
1	13.9	16.3			20.6	22.0		
3	9.4	12.1	20.3	14.3	12.8	15.7	26.9	18.6
6	9.0	11.3			12.9	15.1		
10	9.9	9.5	14.6	10.1	14.1	13.1	19.9	13.4
13	6.1	8.5			9.0	11.9		
17	5.7	8.3	6.7	7.7	8.1	11.4	9.0	10.3
20	9.0	7.8			12.0	10.6		
24	7.4	8.1	8.1	11.1	10.5	12.1	11.0	16.1
27	6.1	5.6			9.3	7.7		
December:								
1	5.7	5.3	8.4	6.9	7.7	7.2	11.4	9.8
4	5.3	8.5			7.8	11.8		
8	5.2	5.3	6.0		7.6	7.6	8.7	
11[a]	9.7	14.7			13.9	20.9		
15	8.4	10.0	9.2		13.5	14.8	16.5	
22	13.9	7.7	12.4		21.1	10.5	18.4	
29[b]	32.4	35.5	46.5		45.8	47.8	62.2	
Average[c]	10.128 ±0.810	11.25 ±0.927						
FROZEN PLANTS								
October:								
23	46.0	62.1			61.7	79.0		
20	61.3	65.3			88.3	94.1		
November:								
1	23.5	44.9			33.8	61.2		
3	53.6	63.6	68.1	53.4	73.8	83.1	89.1	70.5
6	57.1	59.9			81.8	80.7		
10	50.0	55.4	67.1	52.3	71.2	74.5	93.3	69.9
13	11.3	45.4			16.6	63.9		
10	28.8	44.1	56.8	33.3	40.9	60.6	77.8	45.1
20	30.1	41.3			43.8	55.6		
24	44.4	34.5	51.4	50.1	65.3	50.3	70.6	71.5
27	45.6	51.1			67.2	70.5		
December:								
1	33.4	34.3	36.4	26.4	47.1	46.5	50.0	35.9
4	15.4	18.4			22.2	26.5		
8	35.0	28.2	49.1		52.6	39.3	70.9	
11	34.1	43.1			52.5	62.4		
15	28.5	43.3	35.5		43.0	63.5	60.4	
22	41.4	55.7	58.7		60.5	77.7	87.1	
29	42.2	60.0	58.8		62.4	83.6	86.4	
January:								
5	52.4	54.0	55.3		73.7	76.9	83.2	
12	45.9	51.4	50.3		64.9	75.0	73.3	
26	56.3	58.3	59.5		84.3	82.3	79.2	
February:								
12	42.0	55.3	57.1		62.2	82.6	82.5	
23	53.1	51.1	47.2		81.7	70.6	73.2	
March:								
8	41.0	37.6	27.4		73.8	59.0	42.6	
27	24.7	26.4			40.6	46.2		
May:								
17	68.2	69.4			90.8	88.2		
Average	40.973 ±1.820	48.235 ±1.673	51.913 ±1.854					

[a] Samples partly frozen when gathered.
[b] Samples frozen when gathered.
[c] Not including samples collected Dec. 29.

TABLE 5.—*Average quantity of juice expressed, at 339 atmospheres, from duplicate samples of frozen plants of four varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1925–26, University Farm, St. Paul, Minn.*

Date collected	Juice expressed per 100 grams (c. c.)					Percentage of total moisture expressed				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
October:										
12	57.9	69.3	70.9	69.8	70.9	75.4	88.1	87.3	85.3	88.4
16	43.4	62.1	68.7	73.1	57.2	57.6	80.1	85.3	90.4	73.0
20	26.3	37.4	47.6	56.3	25.4	36.3	50.0	60.8	71.8	32.9
24	33.6	44.9	55.6	64.1	37.4	46.6	59.5	70.2	83.2	50.0
28	21.9	30.9	42.7	41.3	28.7	32.6	42.8	58.6	59.4	39.8
31	15.6	24.9	36.6	40.7	9.7	26.4	39.0	57.4	62.3	15.0
November:										
5	10.5	21.7	34.5	47.2	12.9	15.5	30.7	48.6	65.8	17.8
9	18.6	27.5	34.3	34.0	12.3	31.1	43.5	53.2	59.1	18.8
13	32.1	46.1	45.1	45.8	27.9	48.7	65.2	64.0	69.2	39.2
17	20.8	25.5	30.0	33.7	11.8	32.7	37.4	44.1	55.8	17.3
21	26.5	34.8	42.4	46.7	21.3	40.4	50.2	60.8	68.3	30.4
25	12.9	15.0	19.8	29.3	5.7	20.6	22.1	30.2	29.3	8.0
28	25.4	28.4	32.9	29.6	25.5	41.1	42.5	49.6	47.3	36.2
December:										
2	19.7	19.2	20.0	21.7	6.0	31.8	30.4	32.6	36.7	8.9
7	15.0	12.9	20.6	33.8	7.1	21.8	18.8	31.2	52.2	9.2
11	18.2	17.0	30.7	43.2	9.7	27.3	24.4	44.9	56.6	13.3
15	21.7	23.1	38.1	38.8	14.8	34.5	33.6	58.4	63.4	21.3
22	20.1	23.0	23.4	35.6	25.3	32.2	35.0	37.5	55.5	35.5
30	20.5	21.2	25.8	35.1	18.8	32.9	31.8	44.8	59.6	26.7
January:										
7	38.4	44.9			39.8	55.9	63.6			56.1
16	15.1	35.7	40.8	44.7	10.6	22.4	52.0	62.7	67.1	14.6
22	41.1	49.4			35.1	61.4	70.2			51.2
30	41.8	40.4	57.0	47.8	11.6	56.4	57.5	74.9	79.5	16.8
May:										
4	60.8	64.1			66.0	82.7	82.5			83.3
Average [a]	25.961 ±1.630	32.839 ±2.030	38.929 ±2.121	43.443 ±1.907	22.848 ±2.287	38.3	46.5	55.1	62.8	31.3

[a] Not including samples collected May 4.

In the fall and winter of 1925–26, duplicate frozen samples of four varieties of wheat and one variety of rye were tested for the quantity of juice which could be expressed. (See Table 5.) The quantities of juice expressed from the wheat varieties were in inverse order of their hardiness in most of the collections. In many of the tests, however, the quantity of expressed juice from one or more of the wheat varieties was not in the order of the hardiness of those varieties. At the beginning of the season rye yielded more sap than Minhardi wheat, but after October 28 less juice was expressed from rye than from Minhardi, except on five dates. During part of this period less juice was expressed from rye than from any of the wheats. The small quantity of juice expressed from hardened tissues of Minnesota No. 2 rye, which is hardier than any wheat known, shows that hardiness in cereals is largely a matter of imbibition pressure of the cell colloids and tends further to substantiate the results reported by Newton (*34*, *35*, *36*).

It will be noticed that considerable variation occurs in the quantity of juice expressed, even within short periods. Large differences between collections 15 days apart, during a period in which hardening occurred, are shown in Figure 4. Some of this variation between consecutive collections is due, of course, to experimental and sampling

errors and also to differences in technic or treatment of the samples. Most of the variation, however, can be accounted for either by differences in moisture content or by the differences in the water-holding power of the tissues. This latter characteristic could be determined readily by the appearance of the samples removed from the press bowl. The hardened tissues of hardy varieties were not broken down by freezing and pressure, and when removed from the bowl they were elastic and turgid and expanded when the pressure was released; whereas samples of nonhardy or unhardened tissues remained in a thin compact layer when removed from the press bowl. This difference is shown in Figure 7. It was observed that the samples which yielded most of their juice in pressing showed the characteristics of unhardened samples mentioned above, while samples which yielded very little juice were elastic both before and after pressing. Unhardened samples when thawed were of dark color, flaccid, and watery. On several occasions the samples of Minhardi showed as much injury from freezing as Kanred or more.

The differences which exist between plants in adjoining rows or in successive periods, in the ease with which wheat leaves give up water, partly explain why winterkilling in wheat is so variable under different seasonal and environmental conditions. Adjoining rows of wheat often differ noticeably in winter survival, and winterkilling is often irregular even within a short row. Temperature conditions fatal to wheat in one season may not be injurious in another.

In the winter of 1923–24 the increased quantity of expressed juice during December coincided with a warm period in which the growth of the wheat was resumed and the hardening process reversed. The sudden drop in temperature which followed did not permit hardening to occur again before the plants were permanently frozen. The large quantity of expressed juice obtained during the winter of 1924 probably was due to the abnormal seasonal conditions just described. In December, 1925, a gradually increasing quantity of expressed juice again was obtained, this time during continued cold weather. The increased quantity of expressed juice and the apparent coming out from the hardened condition which occurred in this season might be explained, however, in one or both of two ways. Probably some of the tissues were being killed and broken down, thus losing their power of imbibition. The more probable explanation is the increased effect of gradual freezing in the breaking down of protoplasm, mentioned by Bayliss (*6*). The plants were continuously frozen during December but the extent of freezing was increased by subjecting the tissues to the low temperatures of the hardening room. The increased freezing probably removed additional water from the protoplasm to form ice.

It was thought that some of the variation occurring in the samples might be due to methods of handling. One of the variables was the rate and manner of thawing the material. Usually samples were taken from the freezing room to the laboratory and allowed to thaw at room temperature, but occasionally they were placed on or near the radiator to speed up the process. Slight differences were observed in the quantities of juice expressed from the tissue warmed to room temperature or slightly above, when compared with similar samples which were barely thawed.

Samples of rye were collected December 22, 1925, and were subjected to repeated freezings and thawings for different periods. The

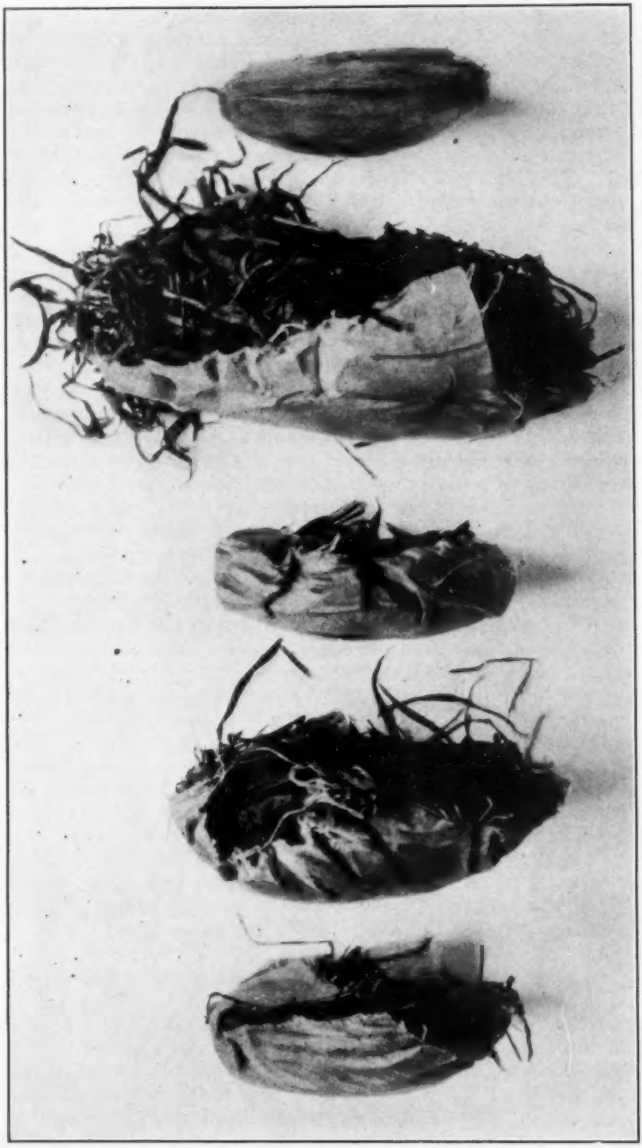

FIG. 7.—Press residues of hardened leaves of wheat and rye plants after being frozen and pressed. Left to right: Kanred, Minhardi, and White Winter wheats, Minnesota No. 2 rye, and Marquis wheat. The hardiness of the varieties is in the direct order of the fluffiness of the samples. Unhardened leaves of the hardy varieties behaved like the Marquis sample

treatments and results shown in Table 6 indicate that thawing to room temperature for a few hours and then freezing again causes only

a slight increase in the quantity of juice expressed. The sample which was frozen and thawed three times yielded only 4.1 c. c. more juice per 100 gm. than the sample thawed but once.

TABLE 6.—*Average quantity of juice expressed, at 339 atmospheres, from duplicate sample plants of Minnesota No. 2 rye collected on December 22, 1925, and subjected to different thawing and freezing periods before pressing, University Farm, St. Paul, Minn.*

Treatment	Date of pressing	Juice per 100 gm.
		C. c.
Thawed and pressed at once	Dec. 29	25.3
Thawed 24 hours at room temperature and pressed	do	28.8
Thawed 6 hours Dec. 29, returned to hardening room, thawed 6 hours, and pressed	Jan. 2	33.9
Thawed at room temperature 6 hours Dec. 29, returned to hardening room; thawed at room temperature 6 hours Jan. 2, returned to hardening room; thawed at room temperature 5 hours and pressed	Jan. 19	29.4

TABLE 7.—*Quantity of juice per gram of dry matter held, after pressing at 339 atmospheres, by frozen and unfrozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923–24, University Farm, St. Paul, Minn.*

Date collected	Water held per gram of dry matter (grams)							
	Frozen plants				Unfrozen plants			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October:								
19					2.36	2.78		
23	1.12	0.77			2.37	3.44		
27	.27	.13			1.76	1.68		
November:								
1	1.52	1.07			1.66	2.22		
3	.69	.55	0.35	0.92	2.44	2.87	2.29	2.70
6	.42	.55			1.99	2.53		
10	.68	.74	.17	.89	2.01	2.28	2.20	2.69
13	1.77	.89			1.90	2.20		
17	1.41	1.06	.60	1.55	2.22	2.39	2.60	2.63
20	1.24	1.28			2.63	2.47		
24	.74	1.09	.79	.67	2.11	1.79	2.52	1.87
27	.69	.78			1.75	2.50		
December:								
1	1.29	1.51	1.34	1.78	2.63	2.56	2.47	2.15
4	1.77	1.67			1.94	2.21		
8	.94	1.54	.66		2.01	2.16	2.02	
11	.88	.84			1.99	1.86		
15	1.12	.78	.57		1.41	1.78	1.05	
22	.85	.57	.27		1.53	2.46	1.70	
29	.78	.42	.40					
January:								
5	.65	.55	.34					
12	.84	.54	.58					
26	.36	.42	.64					
February:								
12	.80	.30	.39					
23	.34	.77	.49					
March:								
8	.34	.72	1.03					
27	.93	.72						
May:								
17	.28	.44						
Average	.874	.706			2.039	2.343		

Duplicate samples of Kanred wheat collected November 4, 1925, during a mist, were left in the laboratory for 20 hours before being placed in the hardening room. These samples had a moisture content only about 2 per cent higher than that of the samples col-

lected the following day and taken to the hardening room immediately, yet they yielded an average of 59.2 c. c. of juice per 100 gm. of tissue, as compared with 21.7 c. c. of juice per 100 gm. of tissue from the latter. Fermentation, which had begun before the first samples were taken to the hardening room, apparently had destroyed the colloidal imbibition of the protoplasm.

A determination of the juice expressed per 100 gm. of fresh tissue does not give an exact measure of the imbibitional capacity of the protoplasm, because the tissue varies in moisture content. The quantity of water held per gram of dry matter after pressing at 339 atmospheres pressure has been calculated, therefore, and the results are presented in Tables 7 and 8. With frozen samples, the placement of varieties or collections is about the same for this relationship as for the quantities of juice expressed per 100 gm. of tissue. On unfrozen samples, however, there is little or no correlation between the quantities of juice held and the hardiness of the varieties. Because of their lower initial moisture content, unfrozen tissues of Minhardi wheat usually held less juice after pressing than those of Kanred.

TABLE 8.—*Average quantity of juice per gram of dry matter held, after pressing at 339 atmospheres, by duplicate samples of frozen plants of four varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1925–26, University Farm, St. Paul, Minn.*

Date collected	Water held per gram of dry matter (grams)				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
October:					
12	0.81	0.44	0.55	0.66	0.47
16	1.30	.68	.61	.68	.98
20	1.68	1.64	1.42	1.03	2.36
24	1.38	1.25	.96	.56	1.49
28	1.40	1.48	1.12	1.14	1.56
31	1.07	1.09	.77	.71	1.58
November:					
5	1.78	1.68	1.26	.88	2.18
9	1.02	.97	.85	.54	1.54
13	1.01	.85	.86	.60	1.50
17	1.19	1.33	1.20	.68	1.87
21	1.14	1.17	.91	.69	1.62
25	1.35	1.67	1.35	.80	2.27
28	1.08	1.17	.99	.88	1.54
December:					
2	1.11	1.25	1.13	.92	1.91
7	1.70	1.78	1.34	.88	2.31
11	1.46	1.75	1.22	.86	2.30
15	1.10	1.49	.78	.58	2.17
22	1.18	1.27	1.04	.73	1.61
30	1.08	1.37	.75	.57	1.76
January:					
7	.98	.88			1.07
16	1.61	1.07	.70	.66	2.29
22	.80	.70			1.11
30	1.24	1.12	.82	.32	1.88
May:					
4	.48	.61			.64
Average	1.206	1.196			1.667

FREEZING-POINT DEPRESSION OF EXPRESSED JUICE

The freezing-point depression of the expressed juice from the frozen plants of wheat and rye was determined in both years of the investigation. The results for 1925–26 are from duplicate samples. The data are presented in Tables 9 and 10 and are shown graphically in Figures 8 and 9.

TABLE 9.—*Freezing-point depressions of the juice expressed from frozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923–24, University Farm, St. Paul, Minn.*

Date collected	Freezing-point depression (°C.)			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October:				
23	1.11	1.18		
27	.87	.80		
November:				
1	1.57	1.47		
3	1.67	1.48	1.47	1.48
6	1.69	1.43		
10	1.68	1.51	1.42	1.38
13	.97	1.01		
17	1.32	1.35	1.30	1.26
20	1.35	1.27		
24	1.14	1.03	1.20	1.10
27	1.71	1.49		
December:				
1	1.60	1.30	1.34	1.20
4	1.42	1.32		
8	1.67	1.51	1.56	
11	2.11	2.03		
15	1.86	1.99	1.97	
22	1.34	1.25	1.18	
29	1.77	1.33	1.38	
January:				
5	1.60	1.53	1.55	
26	2.17	1.85	1.70	
March:				
8	2.16	1.26	.60	
May:				
17	1.30	1.15		
Average	1.549 ±0.050	1.388 ±0.042	1.389 ±0.062	

TABLE 10.—*Average freezing-point depressions of the expressed juice from plants of four varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1925–26, University Farm, St. Paul, Minn.*

Date collected	Freezing-point depression (°C.)				
	Minhardi	Kanred	White Winter	Marquis	Minnesota No. 2 rye
October:					
12	1.25	1.15	1.06	0.99	1.11
16	1.17	1.17	1.03	.99	1.16
24	1.36	1.29	1.24	1.11	1.21
28	1.55	1.42	1.40	1.58	1.50
31	2.21	2.19	2.07	2.15	2.10
November:					
5	.98	1.06	1.13	1.02	.91
9	2.13	2.14	2.01	1.83	2.17
13	1.58	1.43	1.46	1.54	1.37
17	1.70	1.72	1.76	1.96	1.71
21	1.57	1.44	1.51	1.58	1.42
25	1.69	1.62	1.86	2.12	1.41
28	1.90	1.80	1.90	2.17	1.77
December:					
2	2.05	1.80	2.03	1.90	1.66
7	1.14	1.18	1.36	1.47	1.13
11	1.21	1.20	1.29	1.03	1.19
15	1.39	1.36	1.36	1.15	1.37
22	1.99	2.03	2.06	1.19	1.55
30	1.88	1.85	1.93	1.33	1.74
Average (first 18 samples)	1.597	1.552	1.581	1.506	1.471
January:					
7	1.45	1.43			1.29
22	1.47	1.33			1.29
May:					
4	1.11	1.04			1.08
Average [a]	1.584 ±0.052	1.535 ±0.051	1.581 ±0.057	1.506 ±0.066	1.453 ±0.048

[a] Not including samples collected May 4.

The juice of the Minhardi variety had an average freezing-point depression greater than that of Kanred and White Winter in 1923-24, but about the same as White Winter in 1925–26. The juice of rye usually had a lower freezing-point depression than any of the wheats. The freezing-point depression, although fluctuating, tended to increase during the autumn, fluctuated during the winter, and then decreased in the spring after growth was well begun. The freezing-point depressions of the wheat varieties obtained during the first three collections of 1925 and also most of the early collections of 1923 were in the direct order of hardiness. Later in the season the more tender varieties were so low in moisture content that the juice was much more concentrated. The freezing-point depression of winter-wheat juice seems to depend upon the moisture content of the tissue, the quantity of soluble materials elaborated, and the quantity of imbibed water. When the plants are hardened, or partly so, and a complete and representative sample of juice can not be obtained by freezing and pressure, a freezing-point depression of the juice obtained is not a measure of the hardiness of a variety of wheat. When hardening or wilting has not occurred, the freezing-point depression of the plant juice is a fairly reliable measure of hardiness in the wheat varieties studied.

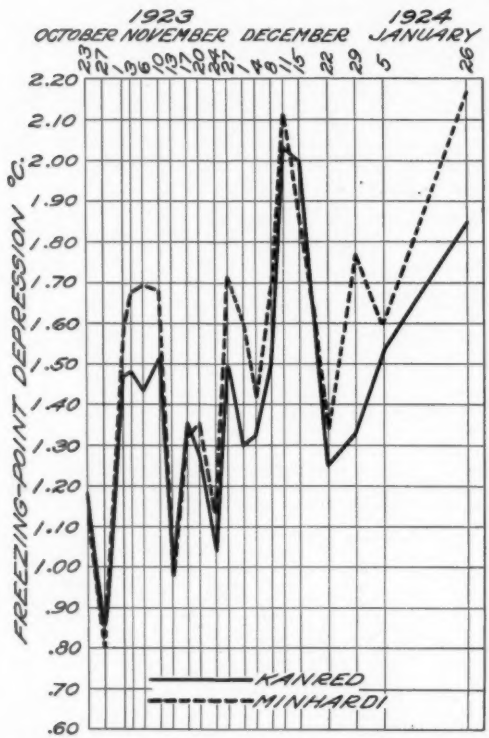

FIG. 8.—Freezing-point depression (°C.) of the juice expressed from the frozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1923–24, University Farm, St. Paul, Minn.

TOTAL SOLIDS IN EXPRESSED JUICE

The percentage of total solids in the expressed juice, as measured by the Abbe refractometer, was determined from both frozen and unfrozen samples in 1923–24. The results are given in Table 11 and are shown graphically in Figure 10. Higher percentages of solids nearly always were present in the juice from frozen plants. Considerable variation existed in the juice from plants collected on different dates, due to variations both in juice concentration and in

the quantities of juice expressed. The percentage of total solids in the juice was higher in Minhardi than in Kanred, and higher in Kanred than in White Winter. The hardier the variety the higher was the content of total solids in the juice on most dates and as an average. Rye juice usually had a lower content of total solids than that of any of the wheats. The total solids determined consisted of material in both molecular and colloidal solution, but probably mostly the former. The higher percentage of total solids in hardy varieties probably is due largely to the lower moisture content, and the increase in total solids during hardening both to decreased moisture content and to the increase in soluble carbohydrates and nitrogen compounds. The higher percentage of total solids in the frozen tissue shows that the disorganization of the protoplasm by freezing causes a release of soluble material not obtained by pressure without freezing. The juice obtained from the unfrozen tissue probably came largely from the vacuoles.

TABLE 11.—*Total solids in the juice, expressed at 339 atmospheres pressure, from unfrozen and frozen plants of three varieties of wheat and one variety of rye collected at intervals during the fall and winter of 1923–24, University Farm, St. Paul, Minn.*

Date collected	Total solids (per cent)							
	Unfrozen samples				Frozen samples			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye	Minhardi	Kanred	White winter	Minnesota No. 2 rye
October:								
19	8.8	9.3						
23	13.6	11.6			11.2	10.0		
27*	3.6	2.7			9.0	7.0		
November:								
1	13.5	8.0			18.0	17.5		
3	13.0	11.0	9.0	10.0	15.0	13.8	12.3	13.5
6	10.5	8.5			16.5	14.0		
10	10.5	10.5	11.0	11.0	16.5	14.3	13.8	13.5
13	9.5	10.0			11.0	10.5		
17	12.0	10.4	12.5	10.0	12.5	12.3	11.2	11.3
20	11.0	11.0			14.5	13.3		
24	8.0	9.5	8.3	8.0	12.0	10.0	11.0	11.0
27	9.5	10.5			16.5	14.5		
December:								
1	9.8	7.0	7.5	5.4	13.0	10.0	10.0	8.0
4	10.0	10.0			10.5	9.0		
8	12.7	11.3	10.0		18.0	14.0	15.0	
11	15.5	13.0			19.5	18.5		
15	19.5	16.5	15.0		18.0	20.0	18.0	
22	14.0	14.0	8.0		13.0	12.0	11.5	
29					15.5	10.0	13.0	
January:								
5					12.3	11.0	12.1	
12					14.8	11.0	10.5	
26					16.5	11.8	11.6	
February:								
12					12.5	12.9	6.5	
26					10.7	8.5	11.5	
March:								
8					18.0	10.0	6.0	
27					11.0	9.5		
May:								
17					4.2	6.3		
Average	11.389 ±0.524	10.267 ±0.447			13.854 ±0.460	11.988 ±0.426	11.600 ±0.496	

* Samples gathered on Oct. 27 contained excess moisture due to snow.

Bound Water in Expressed Juice

The determination of bound water was made in 1923–24 on juice from frozen samples only. The average percentage of bound water in the juice of Minhardi, Kanred, and White Winter varieties varied directly with hardiness. Rye juice showed a bound-water content greater than that of wheat on some dates and less on others. There was extreme irregularity in the quantities of bound water present. The quantity depends both on the binding power of the colloids in the cells and on the quantity of colloidal material which is removed in expressing the juice. Probably the effect of freezing on the precipitation and dispersion of the colloids also has some effect. The plant tissue was not macerated before pressing, so it is unlikely that the colloids in the juice represent the colloidal conditions within the cells. The fluctuations in the percentage of bound water in the juice obtained by the method used render this test of little value in measuring hardiness. The data on bound water are given in Table 12 and are shown graphically in Figure 11.

Table 12.—*Bound water in the juice expressed from the frozen plants of three varieties of wheat and one variety of rye collected at intervals during the autumn and winter of 1923–24, University Farm, St. Paul, Minn.*

Date collected	Bound water (per cent)			
	Minhardi	Kanred	White Winter	Minnesota No. 2 rye
October:				
23	5.8	7.8		
27	6.5	10.0		
November:				
1	0	1.1		
3			1.9	
10	9.7	10.1	8.0	13.9
13		4.7		
17	16.6	7.3	11.1	5.0
20	15.4	13.3		
24	8.3	4.3	2.7	8.0
27	6.5	8.0		
December:				
1	4.7	12.3	5.0	10.4
4	7.3	7.3		
8	17.1	9.4	6.9	
11	14.2	5.0		
15	3.5	1.5	6.9	
22	3.5	2.3	5.0	
29	8.0	10.4	6.9	
January:				
5	3.9	9.7	6.5	
26	7.3	8.0		
March:				
8	16.3	9.0		
May:				
17	11.9	11.6		
Average	8.763 ±0.768	7.655 ±0.516	6.090 ±0.532	

Correlations Between Characters Used to Measure Hardiness

In order to determine the relation of the results obtained in the various tests on the wheat and rye samples, correlation coefficients were calculated. The variables used were from the average of duplicate determinations made in 1925–26 and from single determinations made in 1923–24, except that the moisture-content determinations of

this season also were in duplicate. Data obtained during the entire season were used, thus including data from both hardened and non-hardened plants. The data used in calculating the coefficients were from 15 to 26 experiments in each case, except those on White Winter wheat in 1923–24, of which only 8 to 12 items were available. The correlation coefficients were computed according to the usual formula when using ungrouped variables:

$$r = \frac{\frac{\Sigma xy}{n} - \left(\frac{\Sigma x}{n}\right)\left(\frac{\Sigma y}{n}\right)}{\sqrt{\frac{\Sigma x^2}{n} - \left(\frac{\Sigma x}{n}\right)^2} \times \sqrt{\frac{\Sigma y^2}{n} - \left(\frac{\Sigma y}{n}\right)^2}}$$

when Σ = summation, x = values of one variable, y = values of the other variable, and n = number of observations.

The correlation coefficients obtained are given in Table 13. The quantity of juice expressed is positively correlated, in most cases highly so, with the moisture content of both frozen and unfrozen plants. It is negatively correlated in most cases with freezing-point depression and percentage of total solids in the juice. The freezing-point depression shows a high positive correlation with the percentage of total solids and, especially in 1925–26, a high negative correlation with moisture content. The percentage of total solids is negatively correlated with moisture content. The percentage of bound water does not show any consistent correlations with the other factors measured, the coefficients with Kanred even differing in sign from those of the other two wheat varieties.

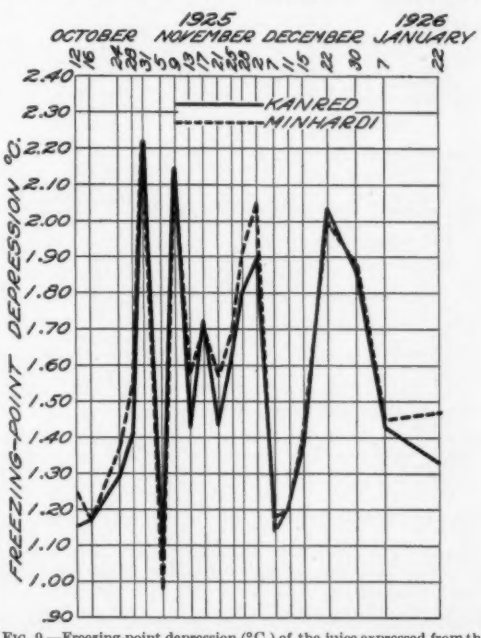

FIG. 9.—Freezing-point depression (°C.) of the juice expressed from the frozen leaves of Kanred and Minhardi wheats collected at interval, during the autumn and winter of 1925–26, University Farm, St. Paul, Minn.

The quantity of juice expressed per 100 gm. of tissue is not an entirely comparable measure when the varieties differ in moisture content. Correlations between the quantity of juice held against freezing and pressure and the moisture content were calculated, therefore, and the coefficients shown in Table 14 were obtained.

TABLE 13.—*Correlation coefficients between physical factors of plants of four varieties of wheat and one variety of rye collected at intervals during the autumns and winters of 1923–24 and 1925–26, University Farm, St. Paul, Minn.*

Factor and variety	Correlation coefficients (r) between—			
	Juice expressed (cubic centimeters per 100 gm.)	Freezing point depression ° C.	Percentage of total solids	Percentage of bound water
Frozen samples, 1923–24				
Moisture content (per cent):				
Minhardi	+0.313±0.119	−0.462±0.113	−0.351±0.116	−0.168±0.150
Kanred	+ .533± .095	− .304± .131	− .008± .132	+ .274± .140
White Winter	+ .654± .100	− .094± .193	− .205± .167	− .192± .205
Juice expressed (cubic centimeters per 100 grams):				
Minhardi		+ .079± .143	− .204± .127	− .017± .155
Kanred		− .102± .142	− .107± .131	+ .151± .147
White Winter		− .405± .163	+ .169± .169	− .045± .213
Freezing-point depression (° C.):				
Minhardi			+ .754± .062	+ .166± .150
Kanred			+ .782± .056	− .276± .139
White Winter			+ .873± .046	+ .160± .208
Total solids (per cent):				
Minhardi				+ .094± .153
Kanred				− .533± .108
White Winter				+ .225± .202
Unfrozen samples, 1923–24				
Moisture content (per cent):				
Minhardi	+ .561± .109		− .336± .141	
Kanred	+ .477± .123		− .110± .156	
White Winter	+ .280± .220		− .634± .143	
Juice expressed (cubic centimeters per 100 grams):				
Minhardi			− .160± .155	
Kanred			− .312± .144	
White Winter			− .191± .230	
Frozen samples, 1925–26				
Moisture content (per cent):				
Rye	+ .717± .068	− .757± .064		
Minhardi	+ .696± .073	− .827± .049		
Kanred	+ .734± .065	− .805± .053		
White Winter	+ .868± .036	− .674± .087		
Marquis	+ .897± .029	− .624± .097		
Juice expressed (cubic centimeters per 100 grams):				
Rye		− .388± .128		
Minhardi		− .310± .136		
Kanred		− .387± .128		
White Winter		− .811± .054		
Marquis		− .670± .088		

TABLE 14.—*Calculated correlation coefficients between the quantity of juice held against freezing and pressure and moisture content of plants of Kanred and Minhardi wheats*

Variety	Correlation coefficients (r)	
	Frozen samples	Unfrozen samples
Kanred	0.088±0.131	0.898±0.030
Minhardi	.112± .131	.916± .026

While the correlation between moisture content and juice held against expression is positive, it is too small in the frozen samples to be significant. With the unfrozen samples, on the other hand, the correlation coefficients of 0.898 and 0.916 indicate that the moisture content probably is the chief factor in determining how much juice can be expressed from the tissues. If the tissues are sufficiently wilted, no juice can be expressed from the samples at the pressures used.

The correlations between the quantity of juice expressed and the moisture content of the frozen samples in 1925–26 are much higher than in 1923–24. It will be observed that the values of the correlation coefficients in the wheats in both seasons are in the inverse order of hardiness. The regression coefficients for the samples of 1925–26 were calculated, and the regression lines for the moisture contents for given quantities of juice expressed are shown in Figure 12. By extrapolating the regression lines somewhat beyond the limits of the data, the approximate moisture content at which no juice could be expressed at 339 atmospheres pressure after freezing is shown. These theoretical moisture contents at which sap can not be expressed are in the direct order of the hardiness of the varieties, except that Kanred and Minhardi are reversed. This shift is the result partly of an occasional larger juice yield from Minhardi than from Kanred late in the season, but chiefly of the lower average moisture content of Minhardi and its ability to thrive at a considerably lower moisture content.

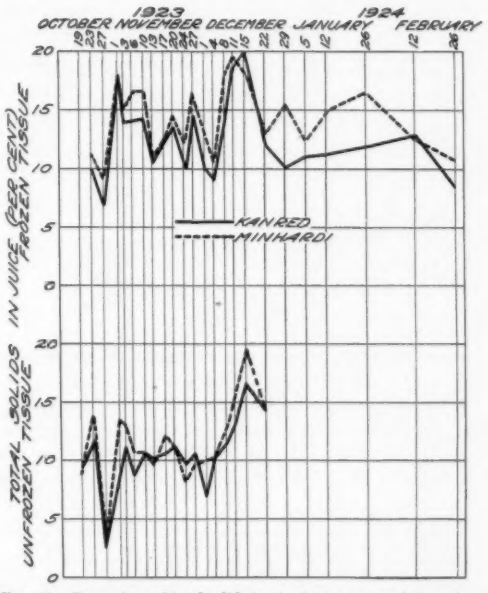

FIG. 10.—Percentage of total solids in the juice expressed from frozen and unfrozen leaves of Kanred and Minhardi wheats collected at intervals during the autumn and winter of 1923–24, at University Farm, St. Paul, Minn.

Partial correlation coefficients were calculated on some of the factors, to compensate for the variation in the moisture content of the samples. These coefficients are given in Table 15. The partial correlation coefficients between juice expressed per 100 gm. of tissue and freezing-point depression are not significantly different from the gross correlation coefficients for 1923–24. Slight positive but not significant partial correlations between juice expressed and bound water were obtained in all three varieties; the higher the correlation the less hardy is the variety. It is still apparent that bound water in

the juice expressed from unground frozen tissue has little direct relation to imbibition pressure in the tissues, but the trend of the partial correlation coefficients indicates that the more the tissue is broken down in freezing and pressing the more colloidal material the juice will contain.

TABLE 15.—*The gross correlation coefficients for juice expressed per 100 gm. of tissue with freezing-point depression and bound water and the partial correlation coefficients (assuming that the moisture content is held constant) obtained on wheat and rye in 1923–24 and 1925–26*

Season and variety	Correlation coefficients for juice expressed per 100 grams of tissue			
	Gross correlation coefficients		Partial correlation coefficients * (moisture content constant)	
	Freezing-point depression (r_{23})	Bound water (r_{24})	Freezing-point depression ($r_{23.1}$)	Bound water ($r_{24.1}$)
Season of 1923-24:				
Minhardi	+0.079	−0.017	+0.265	+0.004
Kanred	− .102	+ .151	+ .198	+ .001
White Winter	− .405	− .045	− .456	+ .109
Season of 1925-26:				
Rye	− .388			+ .340
Minhardi	− .310			+ .641
Kanred	− .387			+ .506
White Winter	− .811			− .616
Marquis	− .670			− .319

* Partial correlation coefficients were computed by the formula: $r_{23.1} = \frac{r_{23} - (r_{12} \times r_{13})}{\sqrt{1-r^2 12}\ \sqrt{1-r^2 13}}$

The nonhardy varieties, Marquis and White Winter, had much higher gross correlations between juice expressed and freezing-point depression than the more hardy varieties, Minhardi, Kanred, and Minnesota No. 2 rye. This showed that the quantity of juice expressed from the less hardy varieties depended more upon the moisture content than in the case of the hardy varieties, and that the lower the moisture content the greater was the freezing-point depression. The hardy varieties, being able to harden, did not yield their juice so completely, as a rule, and thus the concentration or freezing-point depression of the juice varied both with the moisture content of the tissue and in the extent to which the tissue was broken down in freezing and pressing. The partial correlations, assuming moisture content to be constant, still show the freezing-point depression of the juice of the two nonhardy varieties, Marquis and White Winter, to

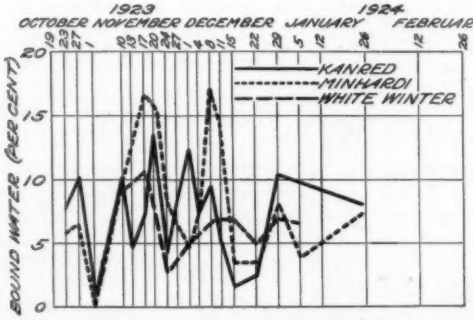

FIG. 11.—Percentage of bound water in the juice expressed from frozen leaves of Kanred, Minhardi, and White Winter wheats collected at intervals during the autumn and winter of 1923–24, University Farm, St. Paul, Minn.

be negatively correlated with the quantity of juice expressed, while in the juice of the three hardy varieties, Minhardi, Kanred, and Minnesota No. 2 rye, these two factors are positively correlated. These results may be merely chance differences, as such relationships were not obtained in the 1923–24 samples. On the other hand, they may indicate that hardy varieties usually yield the more dilute vacuolar sap, but as the protoplasm is broken down, juice which contains more dissolved material is expressed. The nonhardy varieties are almost completely broken down by the freezing at all times, and it appears that the more completely the juice is expressed the more dilute it becomes.

Rate of Respiration at Low Temperatures

The results obtained by Govorov (*18*) indicated that hardy wheats and ryes respired less than nonhardy varieties at low temperatures. Newton (*36*) found that hardy varieties retained their sugars throughout the winter more completely than the nonhardy varieties. Experiments were undertaken by the writer to determine whether these sugars were used up as the result of a greater rate of respiration. Plants of wheat and rye were grown in glazed pots in the greenhouse. Before starting a pot of plants in the respiration experiment, the soil was sealed over with a half-and-half mixture of vaseline and liquid paraffin, which, after melting, could be poured at a temperature of 40° to 45° C. on the soil and around the bases of the plants. The cover was then placed over the plants with its edge resting on the seal inside the pot. The space between the cover and the pot then was sealed. The pots were first placed in the chamber at 5° C., and when the rate of respiration had been determined they were transferred successively to other chambers with temperatures of 0°, −5°, and −10° C., respectively. After the pots had come to a temperature equilibrium in a chamber, 8 to 12 hours being allowed, the CO_2 present was removed with a stream of air from which the CO_2 had been removed, and the plants were alowed to respire. The gradual reduction in temperature permitted the winter varieties to survive the final temperature of −10° C., but the spring wheat Marquis did not survive. The winter varieties showed growth after passing through the respiration experiments and being cut off just above the wax seal. This indicated that the seal used was not toxic. The data obtained in the respiration experiment are presented in Table 16.

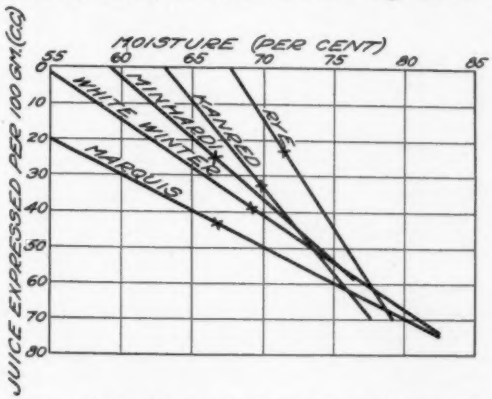

Fig. 12.—Regression lines for the mean values of moisture content plotted against the quantity of juice expressed per 100 gm. of frozen leaves of four varieties of wheat and one variety of rye

TABLE 16.—*Quantity of CO_2 respired by plants of four varieties of wheat and one variety of rye placed successively in chambers at four different temperatures*

Variety and sample	Milligrams of CO_2 respired per hour per kilogram of dry matter at—				Van't-Hoff coefficient −10° to 0° C.
	5° C.	0° C.	−5° C.	−10° C.	
Minhardi 1	126.4	313.0	221.2	66.3	4.7
Minhardi 2	320.5	142.2	163.0	60.8	2.3
Minhardi 3	435.0	322.6	120.9	65.7	4.9
Minhardi Average	294.0	259.3	168.4	64.3	4.0
Kanred 1	351.5	299.5	141.4	88.3	3.5
Kanred 2	323.9	270.6	243.3	185.5	1.4
Kanred 3	258.7	200.2	181.2	92.1	2.2
Kanred Average	311.4	256.8	188.6	122.0	2.4
Red Rock 1	473.5	306.7	219.3	217.1	1.4
Red Rock 2	150.7	37.7	31.3	21.9	1.7
Red Rock 3	357.4	203.2	173.4	49.8	4.1
Red Rock Average	327.2	182.5	141.3	96.3	2.4
Marquis 1	514.9	261.6	143.3	53.0	4.9
Marquis 2	475.0	316.5	225.0	53.9	5.9
Marquis 3	325.5	143.0	193.8	44.0	3.3
Marquis Average	438.5	240.4	187.4	50.3	4.7
Rye 1	672.7	689.6	106.5	30.1	22.9
Rye 2	359.5	433.0	158.5	58.2	7.4
Rye 3	442.8	428.2	232.4	124.0	3.5
Rye Average	491.7	516.9	165.8	70.8	11.3

At the higher temperatures, 5° and 0° C., rye showed the greatest rate of respiration. Marquis, a spring wheat, showed the second greatest rate of respiration at 5°. The low respiration of Minhardi in the first experiment at 5° C. can not be explained. A plant adjustment may have been taking place, or possibly the temperature or air-circulation controls were temporarily out of order and this was not detected. The latter possibility does not seem likely, however, as the quantity of CO_2 respired by the Minhardi plants at 5° C. agreed very closely during two successive periods, indicating an actual depression in respiration.

At the low temperature of −10° C. the quantity of CO_2 obtained per kilogram of dry matter in the winter varieties was in the inverse order of hardiness. The low rate of respiration of Marquis at −10° C. probably was due to the plants having been killed while the winter wheats and rye were not. The Van't-Hoff coefficients calculated for the range of −10° C. to 0° C. show that the increase in respiration between these temperatures was in the order of hardiness of the winter grains. The results, especially those with rye, indicate that hardy varieties are able to conserve their carbohydrates at low temperatures to a much greater extent than are the nonhardy.

DETERMINATION OF LETHAL TEMPERATURES FOR WHEAT

The accidental killing of two of the four varieties of wheat growing in pots in the greenhouse when they were placed outside to harden showed that varieties differed in their ability to withstand low temperatures without previous hardening. Experiments were then begun

to determine the lethal temperatures of wheat and rye taken directly from the greenhouse and also to determine the protection afforded by previous exposure to different low temperatures before subjecting them to temperatures likely to kill. The results obtained are shown in Table 17.

The tests in 1924 were made in the large refrigeration chamber belonging to the division of entomology of the University of Minnesota, and those in 1926 were made in the ice-cream cabinet used for the respiration experiments.

TABLE 17.—*Rye and wheat plants killed by exposure to various temperatures during various periods in 1924 and 1926*

Temperatures (° C.) and duration of exposure	Plants killed (per cent)										
	Minnesota No. 2 rye	Minhardi	Kanred	Fulcaster	Dawson	Red Rock	White Winter	Marquis	Kubanka	Hard Federation	Kanred×Minhardi, F_2 hybrids
RESULTS IN 1924											
−4° 24 hours								0			
−8° 24 hours	0	0	0				96	100			
−10° 24 hours	100	100	100				100				
−12° 24 hours		100	100								
−12° to −15° 24 hours	100	100	100								
−15° 24 hours	100	100	100								
1° 72 hours, −8° 36 hours, −12° 12 hours		50	100								55.3
1° 72 hours, −8° 36 hours, −9° 12 hours		18	6				100				62.6
3° to 6° one month, −10° 24 hours							100				
3° to 6° one month, −15° 24 hours	100	100	100								
RESULTS IN 1926											
−10° 48 hours	100					100					
−7° 24 hours	0		0	50		0	100	100	100		
−5° 24 hours			5				0	100	100	100	
−5° 24 hours (old plants)								0			
−4° 24 hours			0				0	0	0	0	
5° 48 hours, −10° 24 hours		50									
0° 48 hours, −10° 24 hours		20									
−5° 48 hours, −10° 24 hours		80									
0° 48 hours, −5° 24 hours, −7° 48 hours	20	0	39	100	100						
−7° 24 hours (wilted plants)	0		0								
−7° 24 hours (not wilted)	100		100								

All varieties of wheat and rye tested were killed by an exposure for 24 hours to temperatures of −10° C. or lower after being taken directly from the greenhouse. The Minnesota No. 2 rye and the Minhardi and Kanred wheats completely survived temperatures of −8° C. for 24 hours, while 96 per cent of the White Winter and all of the Marquis plants were killed. At −7° C. for 24 hours the rye, Kanred wheat, and one pot of Red Rock wheat survived completely. One lot of Fulcaster was completely killed at the −7° C. temperature, but another lot completely survived although showing severe injury. White Winter, Marquis, and Kubanka were completely killed at −7° C. At −5° C., Kubanka and Hard Federation spring wheats were completely killed. Two lots of Marquis also were

completely killed at this temperature, although one pot containing older plants survived completely at this temperature. White Winter completely survived a temperature of −5° C., as also did all lots of Kanred but one, which showed some injury. Plants of Marquis, which were starting to head, completely survived a temperature of −4° C. for 24 hours. Plants of Marquis, Hard Federation, Kubanka, White Winter, and Kanred, which had not quite reached the jointing stage, completely survived a temperature of −4° C. for 24 hours.

In tests with Minhardi wheat at 0° C. for 48 hours, 80 per cent survived, while at −5° C. for 48 hours only 20 per cent of the plants survived. A previous exposure to 5° C. for 48 hours enabled half of the plants to survive −10° C. for 24 hours. In tests in which the temperatures successively were 0° C. for 48 hours, −5° C. for 24 hours, and −7° C. for 48 hours, 20 per cent of the rye, 39 per cent of the Kanred, and all of the Fulcaster and Dawson, but none of the Minhardi plants, were killed. In other tests, in which the temperatures used were 1° C. for 72 hours, −8° C. for 36 hours, and −12° C. for 12 hours, half of the Minhardi and all of the Kanred plants were killed, while with temperatures of 1° C. for 72 hours, −8° C. for 36 hours, and −9° C. for 12 hours, 18 per cent of the Minhardi plants, 6 per cent of the Kanred plants, and all of the White Winter plants were killed. Plants of White Winter kept for about a month at 3° to 6° C. could not withstand −10° C. for 24 hours, nor could rye, Minhardi, and Kanred, kept under the same conditions, survive −15° C. for 24 hours.

No consistent differences could be observed in the hardiness of wheat seedlings grown in the greenhouse and varying in age from 6 weeks to 4 months.

Plants of Minnesota No. 2 rye and Kanred wheat, which were severely wilted owing to a lack of soil moisture, recovered completely from a freezing of −7° C., while unwilted plants of the same varieties liberally watered were completely killed by this temperature.

HARDINESS OF PLANT PARTS

Plant parts differ in their ability to withstand freezing or desiccation, as shown by Schander and Schaffnit (*50*), Schaffnit (*48*), and Chandler (*9*). When wheat plants are frozen the tips of the oldest leaves show the first injury and wilting, but at extremely low temperatures the entire leaf is wilted. Injury progresses in a given plant from the older to the younger leaves, upon exposure to successive intensities of cold. The same effect occurs in a series of plants each held at one of a series of constant low temperatures. The crown containing the meristematic tissues is the most hardy part of the wheat plant, at least of the portion above the soil surface. Under severe winter conditions all of the wheat leaves die, but if the crown is alive recovery takes place.

In order to determine whether or not all parts of the plants had similar physicochemical properties, different portions of the plants were tested separately on one date in 1923 for moisture content, expressed juice, total solids in the juice, freezing-point depression of the juice, and bound water in the juice, and on two dates in 1925 for moisture content, expressed juice, and freezing-point depression. The results of these determinations are given in Table 18.

TABLE 18.—*Moisture content, juice expressed, total solids in the juice, freezing-point depression of the juice, and bound water in the juice obtained from plant parts of wheat and rye in 1923 and 1925, University Farm, St. Paul, Minn.*

Variety, date of collection, and plant parts	Moisture	Juice expressed per 100 grams at 339 atmospheres	Total solids in juice	Freezing-point depression	Bound water
Minhardi wheat, Dec. 1, 1923:	*Per cent*	*C. c.*	*Per cent*	° *C.*	*Per cent*
Leaves, unfrozen	74.0	5.7	9.8		
Roots and crown, unfrozen	67.2	13.4	6.5		
Leaves, frozen	70.9	33.4	13.0	1.60	4.7
Roots and crown, frozen	65.1	29.9	14.0	1.48	15.7
White Winter wheat, Dec. 1, 1923:					
Leaves, unfrozen	73.6	11.4	7.5		
Roots and crown, unfrozen	67.8	21.1	6.0		
Leaves, frozen	72.8	50.0	10.0	1.34	5.0
Roots and crown, frozen	68.7	37.0	9.0	1.09	12.3
Kanred wheat, Nov. 5, 1925:					
Crown, frozen	70.9	53.5		1.56	
Young leaves, frozen	72.9	55.9		1.59	
Old leaves, frozen	71.3	59.4		1.52	
Minn. No. 2 rye, Nov. 18, 1925:					
Roots, frozen	[a] 79.3	55.9		.58	
Crown, frozen	70.6	26.2		1.44	
Young leaves, frozen	69.8	45.7		1.50	
Old leaves, frozen	70.0	45.3		1.54	

[a] Some wash water on roots, which accounts for higher moisture content, more juice expressed, and lower freezing-point depression.

The frozen samples of the crown and roots of Minhardi and White Winter wheats collected December 1, 1923, contained slightly less moisture than the leaves and yielded correspondingly less juice. The total solids were about the same, and the freezing-point depression of the juice from the crown and roots was about the same as those of the leaves. The chief difference in the two plant portions was in the percentage of bound water. More than three times as much bound water was found in the juice of the crowns and roots as in the leaves of Minhardi, and nearly two and one half times as much in the case of White Winter. Tests for moisture content, quantity of juice expressed, and freezing-point depression, made on the crowns, young leaves, and old leaves of Kanred wheat collected November 5, 1925, yielded very similar results. None of the tissues showed any indication of being especially hardened at this time, although Kanred leaves collected in the forenoon of the same day and left in the laboratory a shorter time yielded less than half as much juice. This indicates that hardening takes place throughout the living portion of the plant and that changes can take place very quickly. The samples of rye collected on November 18, 1925, showed that the crown yielded less juice and consequently was the hardiest portion of the plant. Accurate determinations could not be made on the roots because of the necessity of washing the dirt from the roots with water.

HARDINESS IN WHEAT HYBRIDS

FIELD EXPERIMENTS

The inheritance of hardiness in wheat has been studied by Nilsson-Ehle (*39*) and by Hayes and Garber (*20*). Hybrids were obtained which were hardier than either parent, but the genetic factors involved in hardiness were not determined. Gaines, according to

Schafer (*47*),[6] reported a recessiveness of hardiness in crosses between Turkey (winter) and Jenkin (spring Club) wheats.

The survival of some winter-wheat hybrids in comparison with the parent varieties, the protection of winter wheat obtained by sowing in stubble, and some of the difficulties encountered in determining the hardiness of hybrids in the field will now be considered. Most of these data were obtained in connection with breeding experiments of the western wheat investigations project of the Office of Cereal Crops and Diseases, which were carried on cooperatively at several western field stations. Some results, however, were secured at University Farm, St. Paul, Minn.

The bulked progenies of 36 different crosses, involving combinations of 16 hardy winter-wheat varieties, were grown in the F_3 generation at Dickinson, N. Dak., in 1922 on both fallow and stubble land. Most of the wheat hybrids and parent varieties sown on fallow land were completely winterkilled, and only six crosses showed more than 1 per cent survival. On stubble land the survival of the hybrids ranged from 3 to 100 per cent. The hybrids were mostly intermediate between the parents in their survival, but a few showed survivals approaching the less hardy or the more hardy parent.

In the field the survival of three crosses was observed in detail in the F_3 generation from the progenies of F_2 plants. The cross Kanred × Minhardi was grown at Dickinson, N. Dak., in 1923 and at St. Paul, Minn., in 1926; the Buffum × Eureka cross was grown at Fargo, N. Dak., in 1922 and at Moccasin, Mont., in 1923; and the Minturki × Turkey cross was grown at Dickinson, N. Dak., in 1923. Attempts to study several other crosses were prevented because of either complete killing or of no killing. The number of hybrid lines varied from 99 to 257 in the different tests, in comparison with 11 to 50 parental rows. The hybrid material was all grown under conditions free from winterkilling in the F_1 and F_2 generations. The Kanred × Minhardi hybrids showed survivals approximately intermediate between the parents. The Buffum × Eureka hybrids showed a survival similar to that of the more hardy Buffum parent under the mild conditions at Fargo, N. Dak., in 1922, but under more severe conditions at Moccasin, Mont., in 1923, they were killed as badly as the less hardy Eureka parent.

The Minturki × Turkey hybrid lines survived about as well as the average of the parent varieties. The genetic factors for winter hardiness could not be determined in any of the crosses studied. Much difficulty was encountered in attempting to determine the survival of hybrids in the field, because of the irregularity of winterkilling and the frequency of complete survival or complete killing in several localities.

ARTIFICIAL FREEZING EXPERIMENTS

Artificial freezing was attempted at University Farm, St. Paul, Minn., in order to determine the inheritance of hardiness. Plants were grown in pots in the greenhouse, subjected to low temperatures in a refrigerator, and then returned to the greenhouse and their recovery noted. The results are shown in Table 17.

[6] These inheritance studies were made by E. F. Gaines, cerealist of the Washington Agricultural Experiment Station, in cooperation with the Office of Cereal Crops and Diseases of the United States Department of Agriculture.

In the test involving the series of temperatures, 1° C. for 72 hours, −8° C. for 36 hours, and −12° C. for 12 hours, in which all of the Kanred plants and half of the Minhardi plants were killed, 26 out of 47 or 55.3 per cent, of the F_2 hybrid plants of the cross between these two varieties were killed. This indicated a dominance of hardiness. In the other experiment, under somewhat less severe conditions with successive temperatures of 1° C. for 72 hours, −8° C. for 36 hours, and −9° C. for 12 hours, 62.6 per cent of a total population of 369 F_2 plants were killed. In this test, however, Minhardi was injured somewhat more than Kanred, so the significance of this survival could not be determined.

The only facts regarding the nature of inheritance of hardiness brought out by all of these results are that most of the F_3 strains tend to be intermediate to the parents in hardiness and that the hardier the parents used in the cross are the greater will be the average hardiness of the hybrids. The F_3 and later progenies may show all degrees of survival varying from that of one parent to that of the other, with possibly some transgressive inheritance, as indicated by strains being more or less hardy than either parent. Hardiness in wheat varieties seems to differ over a wide and continuous range. Many varieties doubtless differ genetically in one or more of the factors determining hardiness. It is difficult to find conditions under which one of two varieties of wheat of the same class kills entirely and the other completely survives. Usually both varieties are partly killed, or completely killed, or both survive completely. The extent to which the parents are killed seems largely to determine the extent of killing in their hybrid progeny.

MOISTURE CONTENT OF HYBRIDS

After observing that the difference in moisture content between Kanred and Minhardi wheats existed under nearly all conditions and that moisture content appeared to be related to winter hardiness, it seemed feasible to determine how the moisture difference was inherited.

The material selected for this experiment consisted of the progeny from each of 152 F_2 plants from a Kanred × Minhardi cross grown at University Farm in 1924 in 5-foot rows in conjunction with the parent varieties which occupied adjoining rows distributed at intervals of every eight hybrid rows.

The number of samples which could be gathered, weighed, and dried in the oven within a given period was limited, so that moisture determinations begun on June 3, 1924, were not completed until June 14. Rains occurred during this period, and the moisture content of the samples gathered near its close was slightly higher than that of those gathered at the beginning. The results are shown in Table 19.

The Kanred wheat had an average moisture content of 79.12 ± 0.18 per cent. The Minhardi averaged 76.91 ± 0.21 per cent moisture. The moisture content of the hybrids extended over a range practically the same as that of the two parents combined. The number of rows of both parents and hybrids, however, was not sufficient to give particular significance to the range of variability. The hybrids show a fairly close approximation to a normal frequency distribution when grouped into classes differing in moisture content by 1 per cent. The variability of the parental rows, however, prevents an interpretation

of the mode of inheritance of moisture content in this experiment. The difficulty of determining the inheritance of moisture content, one of the qualities influencing hardiness, the results of which determination were shown in Table 19, tends to show that hardiness itself is not readily analyzed genetically.

TABLE 19.—*Frequency distribution of the moisture contents of plants of Kanred, Minhardi, and Kanred–Minhardi F_3 hybrid winter wheats at University Farm, St. Paul, Minn., in June, 1924*

Moisture content classes	Number of rows		
	Kanred	Minhardi	F_3 hybrid lines
73.0–73.9 per cent			2
74.0–74.9 per cent		2	0
75.0–75.9 per cent		1	10
76.0–76.9 per cent	1	7	32
77.0–77.9 per cent	2	6	33
78.0–78.9 per cent	6	2	35
79.0–79.9 per cent	4	1	30
80.0–80.9 per cent	5		9
81.0–81.9 per cent	1		1
82.0–82.9 per cent			
83.0–83.9 per cent			
84.0–84.9 per cent			
85.0–85.9 per cent			
Total	19	19	152
Mean moisture content	79.12±0.18	76.91±0.21	77.87±0.08

DISCUSSION

The results here presented show many irregularities which, of course, make the drawing of accurate conclusions more difficult than with more uniform data. The studies, however, have brought out many points which must be taken into consideration in formulating the principles involved in hardiness in wheats. Using these studies, as well as the investigations of others, as a basis, the characteristics of hardiness and hardening in wheats and the factors influencing them may be considered.

CHARACTERISTICS OF HARDY VARIETIES

When growing actively in the fall or spring, hardy varieties of wheat as compared with nonhardy varieties are characterized by low moisture content and juice having a high percentage of total solids and a high freezing-point depression. The freezing-point depression and total solids in the juice are closely correlated, and both vary inversely with the moisture content of the tissue. Any variety of winter wheat in an unhardened condition will survive a temperature of −5° C. for 24 hours, but none will survive −10° C. for the same period. A hardening process is necessary, therefore, to permit wheat to survive the low winter temperatures which occur in the Northern States. Wheat plants without snow protection have survived temperatures of −25° C. or lower.

After hardening has occurred, the previously mentioned differences in moisture content and juice concentration between hardy and nonhardy varieties may not hold, or the relationship may be reversed. The hardy and nonhardy varieties then differ chiefly in imbibitional pressure of the cell colloids, as determined by the ability of wheat leaves to hold their moisture against the forces of

freezing and pressure. Hardy rye in an unhardened condition has a moisture content and juice concentration similar to those of a nonhardy wheat, but when hardened it has a greater imbibition pressure than the hardiest wheat.

At low temperatures, hardy varieties of rye and wheat have a lower rate of respiration than nonhardy wheats and thus retain their sugars longer or to a greater extent.

CHANGES OCCURRING DURING HARDENING

When growth is retarded by low temperature the sap concentration increases, due to the formation of sugars from starch and, after freezing, to the splitting of proteins into amino acids, as shown by Newton.[7] The sugars and some salts partly protect the protoplasmic colloids from being coagulated by freezing, according to Newton[7] (*35*, *36*) Maximow (*27*), Schaffnit (*48*), and Schander and Schaffnit (*50*). Freezing of the plants causes water to be withdrawn from the cells. Harvey (*19*) found that on thawing much of this water is evaporated, leaving the cell sap more concentrated than before freezing. Thus the moisture content of wheat leaves decreases during the autumn. With a decrease in moisture content and an increase in sugars and amino acids, the total solids increase and the freezing point of the juice is lowered. This lowering of the freezing point is of little consequence in itself, but the increase in solutes decreases the formation of ice and may protect the protoplasm from precipitation.

When the sap concentration increases, the resistance to freezing of the plant tissue increases at a still greater rate, as noted by Chandler (*9*). Apparently the increased juice concentration results in an increased imbibition pressure of the cell colloids, which decreases the withdrawal of water from the cells by freezing or pressure. Probably the increased imbibition pressure is the result of greater dispersion of the protoplasmic proteins. According to Petit (*41*), imbibed or capillary water permits more undercooling before ice is formed.

Hardy varieties having a greater initial juice concentration possess a slightly greater resistance to freezing injury, probably because less water is withdrawn from the cell in freezing. At low temperatures, hardy varieties form more sugar and increase the sap concentration more than nonhardy varieties. More of the plant tissues of hardy varieties also remain active after a freeze, even with low moisture content. The greater juice concentration in the living cells of the hardy plants causes a greater imbibition of cell juice by protoplasmic colloids. This, together with the greater protection against coagulation of the protoplasm by freezing, due to sugars, accounts for the cold resistance of certain varieties of wheat and rye. The more hardy the variety the more readily is it able to increase the imbibition pressure of the tissues.

The temperatures or combination of temperatures best suited for hardening in wheat are not known, and their determination should furnish a fertile subject for investigation. Results reported here show that gradual reductions in temperature increase hardiness and that exposure to 0° C. is more effective than exposure to temperatures

[7] NEWTON, R. Op. cit.

of 5° C. or −5° C. Probably alternations of freezing and thawing are most effective.

The time required for maximum hardening also is not known. Rein (*42*) reported that maximum hardening occurred in some plants in 6 to 8 days. Winkler (*57*) reported that trees can be hardened by a 5-day exposure to low temperature even in the summer.

When the temperature rises above freezing, water can enter the plants and also the cells if the protoplasm has not been killed. This increase in moisture decreases the juice concentration. At a sufficiently high temperature the sugars will revert to starch and the amino acids will form proteins. The imbibition pressure will then decrease, and the tissues will be less hardy. Rather marked differences have been observed between successive collections made three or four days apart. A decided difference in rye was observed between the collections of November 17 and 18, 1925, and a still more abrupt difference was observed between the Kanred wheat collected in the morning and that collected in the afternoon of November 5. However, the later collections of both rye and wheat were in the laboratory three or four hours while the plant parts were being separated, which might account for some of the changes in these instances.

A freeze during the night, followed by thawing, will increase the quantity of juice that can be expressed from the leaves. After the evaporation of the melted ice crystals, less juice can be expressed. If a freeze is not too severe, the plant hardens upon thawing, but after a hard freeze the protoplasm is coagulated and dehydrated to such an extent that it can not take up water and the plant is killed instead of hardened.

With different freezing temperatures affecting hardy and nonhardy varieties somewhat differently, and with various degrees of recovery or response to temperatures above freezing, the irregularity in the winterkilling of wheats is not surprising. Adjoining rows of wheat show rather wide differences in the imbibition pressure of the tissues. Doubtless there are nearly as great differences between plants within a row. If so, this further explains the irregularities in killing which usually occur and which can not be explained by differences in protection of the plants.

THE PRACTICAL MEASUREMENT OF HARDINESS

The results of these studies indicate that no laboratory method yet devised, except perhaps the controlled freezing, is any more accurate for determining hardiness than is careful field study. The moisture content of unwilted wheat leaves probably is only one of the factors concerned in hardiness and is itself subject to considerable variation. When wilted tissue is present, moisture content is not a dependable character, as tender varieties are wilted more than hardy ones. Under ideal conditions, however, the moisture content is a valuable index of hardiness in wheat leaves.

The imbibition pressure in wheat leaves may show a striking relationship to hardiness during late autumn, but even then it is highly variable from day to day. When the tissue remains frozen during the winter, or when the plants freeze while in a growing condition, the imbibition pressure, as measured by the quantity of expressed juice, is of little value in determining hardiness. This test requires

considerable labor and equipment at all times; therefore, in view of its variability, it can not be expected to serve as a practical measure of hardiness in wheat except under ideal conditions such as are obtained in certain seasons.

Total solids, freezing-point depression, and bound water in the juice are related to hardiness only under certain conditions and are of little use for practical measurement of hardiness except early in the autumn, when the total solids and freezing-point depression are inversely proportional to hardiness. The bound-water content of the juice from macerated tissue should measure the imbibition of the protoplasmic colloids, but its determination is more tedious than that of ordinary freezing and pressing.

Freezing under controlled temperatures offers the greatest promise in measuring the hardiness of wheat plants by laboratory methods, even though the cold resistance of the plants does vary considerably. A rough measure of hardiness can be obtained without any previous hardening, but for more accurate measurements the plants must be subjected to successively lower temperatures. Refrigeration equipment, although expensive, probably would be the most economical means of accurately measuring cold resistance, if an exact technic in hardening were developed. The next most feasible method is by growing the varieties in replicated rows at selected field stations. The plants can be protected somewhat by sowing the seed between rows of grain stubble where conditions are rather severe, or protection can be decreased by thin sowing.

SUMMARY

The studies here presented are concerned only with low temperatures and not with killing due to winter drought, soil blowing, heaving, or smothering by ice.

Hardy wheats are characterized by a low moisture content of the tissues, a high percentage of total solids in the juice, a high freezing-point depression or osmotic concentration of the juice when the plants are actively growing, a high percentage of bound water in the juice, a low rate of respiration at low temperatures, and frequently by a long period of vegetative growth.

The most important character influencing hardiness is the ability to build up a high imbibition pressure of the cell colloids during hardening.

Swedish (Minnesota No. 2) rye, which is more hardy than any wheat, has a high moisture content, a low percentage of total solids, and a low freezing-point depression like nonhardy wheats, but it has a lower rate of respiration at low temperatures and a greater imbibition pressure than the hardiest wheats.

During the hardening of wheats there is a decrease in moisture content and an increase in total solids in the sap, freezing-point depression of the sap, and imbibition pressure of the cell colloids, as measured by the ability of the tissues to hold sap against the forces of freezing and pressure.

In the tissues of a given variety, the quantity of juice expressed is positively correlated with the moisture content. The percentage of total solids and the freezing-point depression of the juice are negatively correlated with moisture content and the quantity of

juice expressed from the tissues. The quantity of juice retained after pressing is positively correlated with moisture content in unfrozen tissues, but not significantly so in frozen tissues.

The moisture content, sap concentration, imbibition pressure, and hardiness of wheat and rye plants fluctuate widely during the fall and winter, and differences between varieties do not exist at all times.

The freezing of plants at controlled temperatures, followed by a determination of the extent of killing, appears to be the most feasible laboratory method for measuring hardiness.

A sudden exposure of wheat and rye plants from the greenhouse to a temperature of $-10°$ C. for 24 hours will kill the hardiest varieties. Any variety of winter wheat will withstand a temperature of $-5°$ C. for the same period. Hardening is necessary to protect wheat against the much lower temperatures than $-10°$ C. which occur in northern wheat-growing areas. The hardier the variety the more it can harden.

The crown is the most hardy portion of the wheat plant above the soil surface. Young leaves are more hardy than old leaves, and the bases of leaves are more hardy than the tips.

The genetic factors involved in the inheritance of hardiness have not been determined, but most F_3 strains are intermediate between the parents in hardiness. Accurate measures of hardiness in the field are difficult because of the variations both in the plants and in weather conditions.

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THE SEEDS OF QUACK GRASS AND CERTAIN WHEAT GRASSES COMPARED [1]

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INTRODUCTION

Because of the great damage to farm land caused by quack grass in northern and eastern United States, it is important that the seed analyst be able to identify positively quack grass seed [2] when it occurs in forage crop seed. Occurring as an impurity in seeds of the coarser grasses and especially in seed of awnless brome grass (*Bromus inermis* Leyss), quack grass seeds are easily confused with seeds of western wheat grass (*Agropyron smithii* Rydb.) and slender wheat grass (*Agropyron tenerum* Vasey). Descriptions of the seeds of the three species of Agropyron have already been published by Pammel and King, Sarvis, Bolley, and Dahlberg.[3] Hillman [4] has described the spikelets in detail as they appear in commercial lots of seed. Sarvis has contributed a key to the seeds based on the pubescence and glands on the palea and rachilla segment. Dahlberg, in the most comprehensive paper heretofore published on the subject, has described briefly the differences in general form, in the shape of the rachilla segment and the outline of the palea tip, and has compared the number and kind of hairs on the rachilla segment, palea, and lemma.

Nothing need be added to what has already been written concerning the pubescence on the seeds of these three species, but for the benefit of those not equipped with magnifiers of sufficient power (35 diameters or more) more consideration may be given to the differences in general form of the seed and to the differences in form exhibited by the different parts of the seed. Frequently an examination of the structural characters is sufficient to determine the species.

The writers describing individual seeds of the three species state clearly that there is no one character that can be relied upon as a means of identification. This is necessarily true because the characters which have to do with quantity and kind of hairs vary greatly, even between individual seeds of the same sample, and the characters, such as shape of rachilla segment and outline or shape of seed, depend somewhat on the maturity and the position of the seed in the spikelet. A determination of species requires familiarity on the part of the analyst with the variations both in pubescence and in general form.

[1] Received for publication Apr. 28, 1927; issued November, 1927.

[2] The word "seed" in this paper is used in the popular sense and refers to the ripened floret.

[3] BOLLEY, H. L. WORK DONE UNDER THE SEED CONTROL ACT (PURE SEED LAW). N. Dak. Agr. Col. Bien. Rpt. 10 (1): 93–118. 1910.

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It is the purpose of this paper to point out graphically the differences in form and appearance as exhibited by the seed as a whole and by its component parts. For the convenience of the analyst, an illustrated tabulation of the differences in pubescence as described by Dahlberg, together with explanatory text, is also given.

In the absence of a single constant diagnostic character, those characters which are displayed in the greatest number of seeds of a species are the ones a study of which will prove most helpful. Familiarity with all of the characters of a species and the corresponding characters in the other species will enable one to identify the average seed.

In addition to the characters generally true of a species, there are others to be found in occasional seeds, the counterpart of which can not be found in the seed of the other two species. A knowledge of these extremes in form peculiar to one of the three species is also helpful.

CHARACTERS GENERALLY TRUE OF A SPECIES

GENERAL FORM

Seeds of *Agropyron repens* are narrowly boat shaped in outline and acutely pointed at the apex. They are thick, somewhat flattened dorsoventrally, with a ventral groove the entire length of the grain. The palea in conforming to the shape of the grain is broadly concave throughout its entire length.

Seeds of *Agropyron smithii* resemble those of *A. repens* in outline, but are more robust and are not flattened dorsoventrally at the callus to the degree evident in seeds of *A. repens*. The palea is more deeply concave.

Of the three species here compared, the seeds of *Agropyron tenerum* are the most easily recognized by their general form. They differ from seeds of *A. repens* and *A. smithii* in having their greatest width at a point a little above the middle and in having a much thinner grain, the ventral groove becoming very shallow toward the apex. This causes the palea to flatten out at the tip.

The bilateral displacement of the lemma and the lack of symmetry observed in many seeds of *Agropyron tenerum* also serve to differentiate a large proportion of the seeds of this species from those of the other two. The lack of symmetry is due to the unequal bending of the different parts of the floret to conform to the closely appressed position of the spikelet on the stem. The displacement of the lemma is a result of the curving of the upper portion of the lemma toward the stem of the spike, while the palea, being shorter and adherent to the rigid grain, remains straight. (Fig. 1.)

COLOR AND TEXTURE

Seeds of *Agropyron repens* found in brome-grass samples are generally light yellow tinged with green, while the seeds taken from well-matured spikes have a brownish tinge, and sometimes are a darker shade above the callus on the dorsal side. The palea and the lemma are thick and lustrous.

Mature seeds of *Agropyron smithii* often are deeper yellow and the lemma and palea sufficiently thin to show the dark-colored grain. In fresh unrubbed seed, the surface of the lemma and palea is not lustrous as in the other two species.

The lemma and palea of *Agropyron tenerum* are pale flesh-colored, thin, and polished, and show very little of the greenish color so prevalent in seeds of *A. repens*.

TIP OF PALEA

The outline of the tip of the palea is worthy of consideration even though in *Agropyron repens* and *A. tenerum* there is considerable variation.

In *Agropyron repens* the tip of the palea is usually truncate or indented, occasionally obtuse or rounded. The broadly truncate type has not been observed by the writer in either of the other two species.

The tip of the palea of *Agropyron smithii* is either indented or cleft. The deeply cleft tip which is frequently found in this species is rarely found in the other two species.

In *Agropyron tenerum* the tip of the palea is either narrowly truncate, rounded, or indented. (Fig. 2.)

RACHILLA SEGMENT

While the length and breadth of the rachilla segment vary within the species according to the position of the seed in the spikelet, the divergence of the sides of the rachilla segment keeps within certain limits for each species and when considered with other characters serves to identify the seed.

The sides of the rachilla segment of *Agropyron repens* diverge slightly in the lower seeds of the spikelet, but in those taken from the middle and upper part of the spikelet the sides of the rachilla segment are approximately parallel. In all seeds the sides of the rachilla segment are straight, and the segment appears flattened against the palea, either within the inturned keels or against them.

The sides of the rachilla segment of *Agropyron smithii* diverge noticeably except in some terminal seeds, and the lines of divergence, instead of being straight as in the other two species often curve out at the upper end. The segment is not appressed to the palea.

The sides of the rachilla segment of *Agropyron tenerum* diverge slightly in all except terminal seeds. The rachilla segment is not appressed to the palea, and in many seeds the segment is inclined laterally following the slight curvature of the seed. The long hairs on the rachilla segment of *Agropyron tenerum*, visible with a hand lens, serve to distinguish a large proportion of the seeds of this species. (Figs. 2 and 3.)

LEMMA

It will be noted that in well-developed seeds of *Agropyron repens*, with the exception of those developed from basal florets, the marginal areas of the paleas are not wholly covered by the lemma. In the seeds of the other two species, the lemma generally extends to the keels of the palea. (Fig. 1 and 2.)

Examination of the sinus, or the opening between the edges of the lemma at the base of the rachilla, is helpful in distinguishing the seeds of *Agropyron repens* from those of the other two species.

In *Agropyron repens* the sinus is essentially U-shaped, the edges of the lemma not obscuring or overlapping the sides of the rachilla segment at its base.

The sinus in *Agropyron smithii* and *Agropyron tenerum* is more V-shaped and the edges of the lemma at the callus obscure the sides of the rachilla segment.

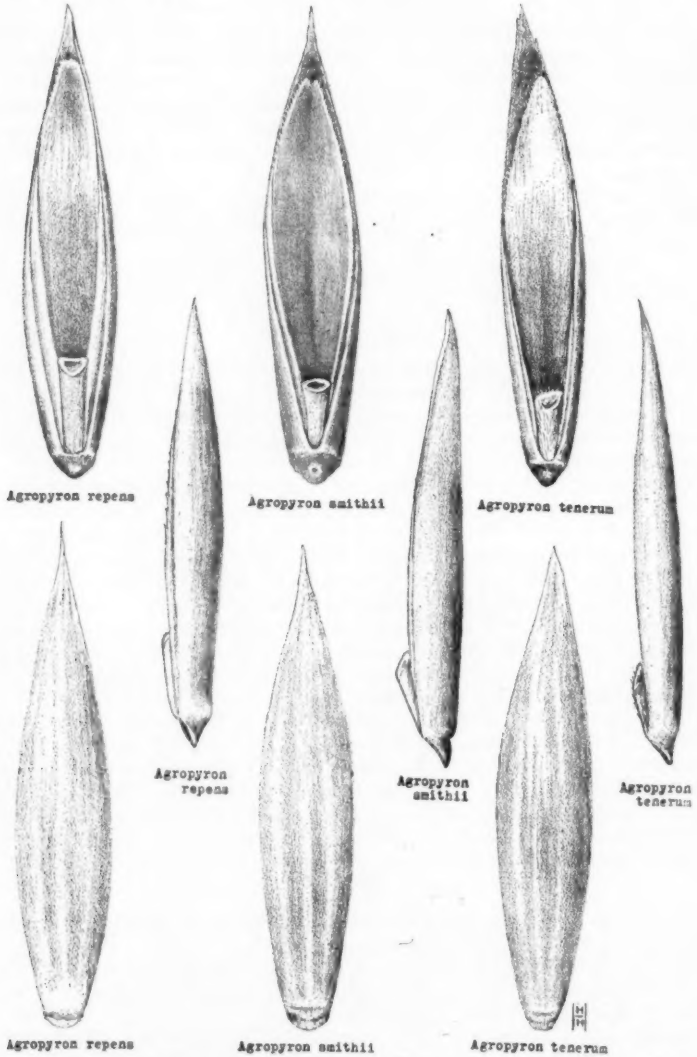

FIG 1.—Dorsal, lateral, and ventral view of the seeds of three species of Agropyron. × 7½

In some basal seeds of *Agropyron smithii* the sinus is so narrow and the rachilla segment so short that only the top of the segment is visible. (Fig. 3.)

FREE GRAINS

The free grains of the three species resemble one another closely, and there is no way known to the writer by which the species can be determined. Grains of the three species are generally light brown,

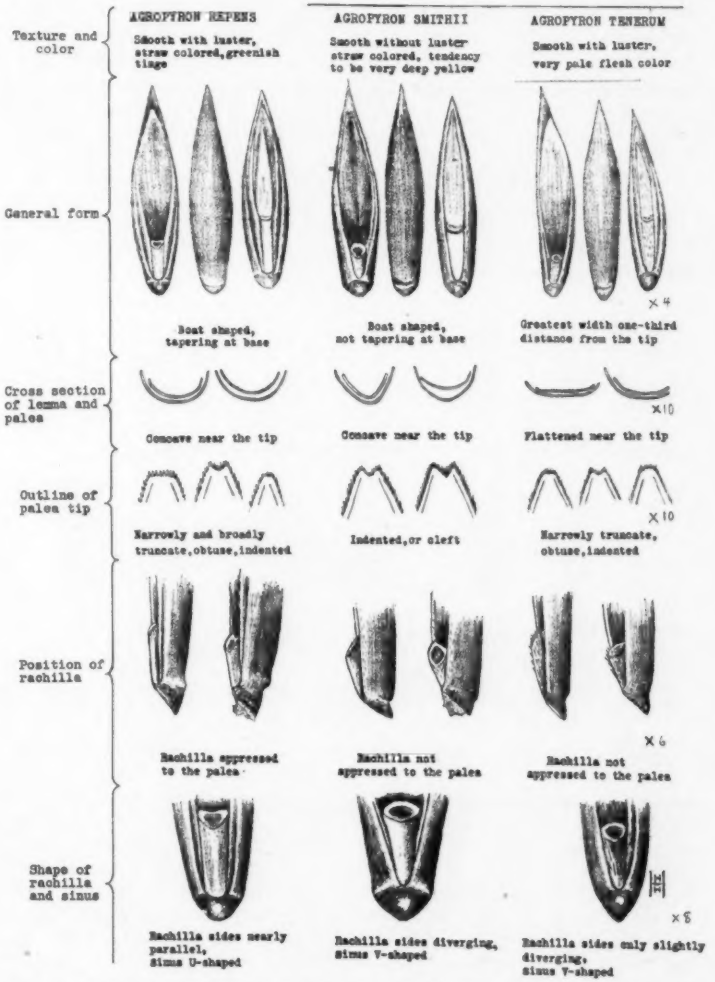
AGROPYRON REPENS
AGROPYRON SMITHII
AGROPYRON TENERUM
Texture and color
Smooth with luster, straw colored, greenish tinge
Smooth without luster straw colored, tendency to be very deep yellow
Smooth with luster, very pale flesh color
General form
×4
Boat shaped, tapering at base
Boat shaped, not tapering at base
Greatest width one-third distance from the tip
Cross section of lemma and palea
×10
Concave near the tip
Concave near the tip
Flattened near the tip
Outline of palea tip
×10
Narrowly and broadly truncate, obtuse, indented
Indented, or cleft
Narrowly truncate, obtuse, indented
Position of rachilla
×6
Rachilla appressed to the palea
Rachilla not appressed to the palea
Rachilla not appressed to the palea
Shape of rachilla and sinus
×8
Rachilla sides nearly parallel, Sinus U-shaped
Rachilla sides diverging, Sinus V-shaped
Rachilla sides only slightly diverging, Sinus V-shaped

FIG. 2.—Diagnostic characters of the seeds of three species of Agropyron, showing differences in general form and appearance

those of *Agropyron smithii* being sometimes very deep purple. The grains of *Agropyron tenerum* are thinner and longer than those of the other two species, and the ventral groove becomes only slightly concave at the upper end.

For the convenience of the analyst, the diagnostic characters which are generally true of each species are listed in an illustrated tabulation (fig. 2).

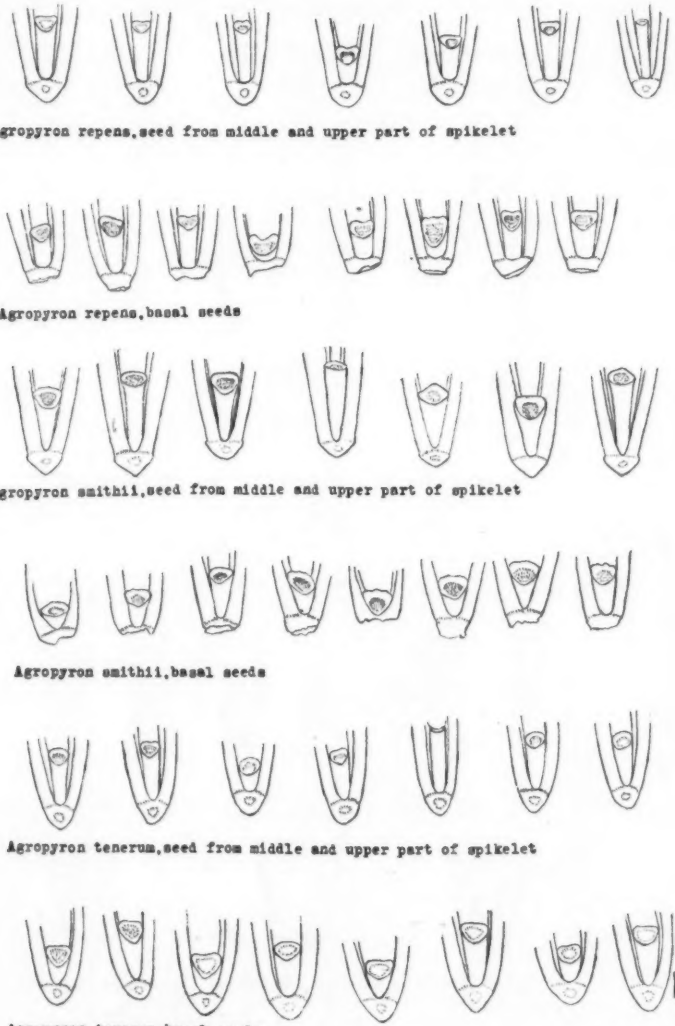
Agropyron repens, seed from middle and upper part of spikelet
Agropyron repens, basal seeds
Agropyron smithii, seed from middle and upper part of spikelet
Agropyron smithii, basal seeds
Agropyron tenerum, seed from middle and upper part of spikelet
Agropyron tenerum, basal seeds

FIG. 3.—Basal portions of the seeds of three species of Agropyron, showing the position of the lemma in relation to the rachilla

CHARACTERS AS MODIFIED BY IMMATURITY AND POSITION OF SEED IN SPIKELET

The ease with which the individual seeds of the three species can be determined depends somewhat on the maturity of the seeds and whether or not the seeds represent terminal florets.

Terminal seeds are difficult to determine as to species because of the straplike elongation of the rachilla and the shortening of the lemma. When immature, the seeds of the three species resemble one another so closely that an examination of the pubescence is necessary for a safe determination.

Seeds developed from basal florets when found free or inclosed in the glumes are easily identified. Although the rachilla is shorter and the lemma longer than in seeds from the upper part of the spikelet, the diagnostic characters are clearly evident. A knowledge of the differences in the glumes described by F. H. Hillman [5] enables one to identify easily seeds inclosed in the glumes. Because of the constant adherence to the stem of the spikelet, the lowest floret of *Agropyron repens* and *A. smithii*, when found free, generally shows evidence of being torn at the callus.

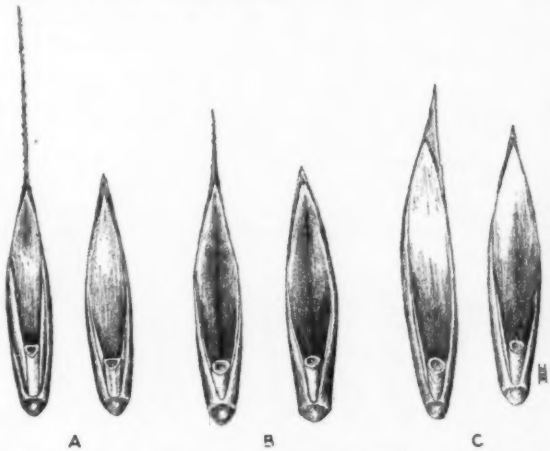

FIG. 4.—Seeds of three species of Agropyron, showing length of awn and acutely pointed lemmas: A, *Agropyron repens;* B, *A. smithii;* C, *A. tenerum.* × 4

CHARACTERS POSSESSED BY OCCASIONAL SEEDS OF A SPECIES

The characters exhibited by occasional seeds of *Agropyron repens* which are not duplicated in seeds of *A. smithii* and *A. tenerum* are the long awn, the longitudinal wrinkle in the palea, and the pronounced bulge at the base of the lemma.

Seeds of the long-awned form of *Agropyron repens* are found frequently in awnless brome-grass seed. Figure 4 shows the long-awned form of *A. repens* and the acuminately tipped lemmas of *A. smithii* and *A. tenerum*. Botanists have reported a long-awned form of *A. tenerum* growing in high altitudes in the Colorado Rockies, but it is very improbable that seeds of this plant would ever occur in trade samples. Seeds resembling those of *A. tenerum*, having a very long awn, frequently have been found in awnless brome-grass seed from Canada. These have been identified as *A. caninum* (L.) Beauv., a closely allied species. Aside from the long awn, seeds of

[5] HILLMAN, F. H. Op. cit.

A. caninum differ from those of *A. tenerum* in having longer hairs, both on the rachilla and around the callus.

The longitudinal fold or wrinkle in the palea is shown in Figure 5. Seeds of *Agropyron repens* having this wrinkled palea are not of frequent occurrence in forage-crop seed, but a sample of *A. repens* recently examined contained a considerable number of seeds of this type. All having this type of palea were well-developed seeds.

The pronounced bulge at the base of the lemma just above the callus, observed in some well-developed seeds of *Agropyron repens*, is shown in side profile in Figure 6, with the corresponding view of the seeds of the other two species. Not only is the bulge less conspicuous in *A. smithii* and *A. tenerum*, but the back edge of the callus is very nearly in line with the back of the lemma, while in the seeds of *A. repens*, figured, the back edge of the callus is coincident with the vertical axis of the seed. In the seeds of *A. repens*, here figured, and occasionally in seeds of *A. tenerum*, the lemma is not indented below the bulge. This is in marked contrast to the lemma of *A. smithii*, which apparently always is abruptly indented or broken at this point. (Fig. 6.)

A character observed in seeds of *Agropyron smithii* and apparently not appearing in seeds of *A. repens* or *A. tenerum* is the deep groove in the deeply concave palea.

FIG. 5.—Seed of *Agropyron repens*, showing wrinkled palea. × 7½

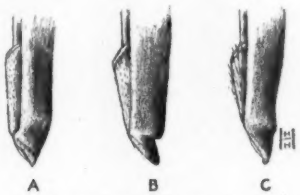

FIG. 6.—Side view of basal portion of seeds: A, *Agropyron repens;* B, *A. smithii;* C, *A. tenerum*. × 7

FIG. 7.—Seed of *Agropyron smithii*, showing deep groove in palea. × 7

Figure 7 shows a seed of this type taken from the upper part of the spikelet. These should not be confused with seeds of *A. tenerum*, which have a shallow groove in a broadly concave palea.

DIFFERENCES IN PUBESCENCE AS DESCRIBED BY DAHLBERG

Figure 8 shows the differences in pubescence, as described by Dahlberg.[6] The following is from his paper, his figures and references to them being omitted:

RACHILLA

The hairs clothing the rachilla constitute a valuable character used in the determination of the seed. However, care and good judgment must be exercised because of the great variation which may occur.

The characteristic rachilla of *A. repens* is sparsely covered with short, minute hairs having a rather large base. Occasionally a glandular structure may be

[6] DAHLBERG, R. C. IDENTIFICATION OF THE SEEDS OF SPECIES OF AGROPYRON. Jour. Agr. Research 3; 275-282, illus. 1914.

discerned at the base. This, however, can only be seen with a high-power lens and is not considered of sufficient importance to warrant its use as a determining character. No rachilla of *A. repens* has been found which had the hirsute character of *A. smithii* or the pilose character of *A. tenerum*.

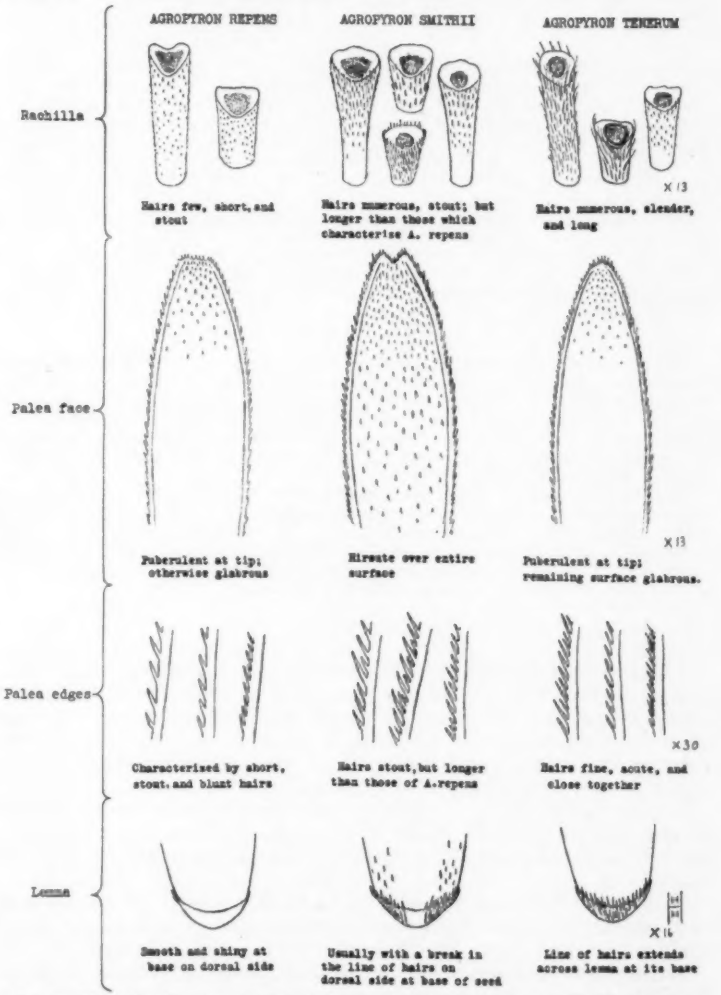
AGROPYRON REPENS
AGROPYRON SMITHII
AGROPYRON TENERUM
Rachilla
Hairs few, short, and stout
Hairs numerous, stout; but longer than those which characterize A. repens
Hairs numerous, slender, and long
X 13
Palea face
Puberulent at tip; otherwise glabrous
Hirsute over entire surface
Puberulent at tip; remaining surface glabrous.
X 13
Palea edges
Characterized by short, stout, and blunt hairs
Hairs stout, but longer than those of A. repens
Hairs fine, acute, and close together
X 30
Lemma
Smooth and shiny at base on dorsal side
Usually with a break in the line of hairs on dorsal side at base of seed
Line of hairs extends across lemma at its base
X 14

FIG. 8.—Diagnostic characters of seeds of three species of Agropyron, showing differences in pubescence, as described by Dahlberg

The rachilla of *A. smithii* is characterized by hairs of the same general shape as the hairs found on the rachilla of *A. repens*. They are, however, larger and stronger and the number is noticeably greater. This characteristic is fairly uniform.

The rachilla of *A. tenerum* is characterized by hairs of a pilose nature. They are long as compared with those of *A. repens* and *A. smithii*, and may often be

distinguished by this feature alone from these two species, as the pilose nature has never been observed on them. However, an absolutely authentic specimen of *A. tenerum* has been examined which had a rachilla much resembling that of *A. repens*. The hairs were short, but were not as large as the base. Other characters on the seed, however, made it possible to place it accurately in the species *tenerum*.

LEMMA

Another distinguishing character and one which is reliable as to uniformity may be found at the base of the lemma on the ventral[7] side of the seed. In *A. tenerum* there is a line of hairs which extends from the base of the rachilla on the dorsal side of the seed around and entirely across the face of the lemma on the ventral side near the base of the seed. In some cases it may be impossible to distinguish the hairs on the middle of the lemma, but the surface of the lemma at this point is roughened sufficiently so that it is noticeable. This is a fairly definite character.

The seed of *A. repens* has no such characteristic line of hairs, but the basal portion of the lemma is entirely smooth and shiny. This character in the seed of *A. smithii* is somewhat variable and is therefore not of much value. Most commonly, however, it is found that the ring of hairs extends part way around on either side, and on the middle of the lemma there is a space which usually is entirely smooth.

PALEA

The part of the seed which discloses good and reasonably definite characteristic differences is the palea. The face of the palea in *A. repens* and *A. tenerum* is practically glabrous, except near the tip, where it is puberulent. Occasionally there is a small number of hairs distributed over the face of the palea. Since the tips of the palae in both of these species are always puberulent, this can not be used as a distinguishing character. The palea of the seed of *A. smithii* is quite hirsute over its entire surface.

The hairs on the edge of the palea have a distinctive shape for each of the three species and are very useful as a determining factor. Those of *A. repens* are rather short, stout, and somewhat blunt. Those of *A. smithii* are about as coarse as those on *A. repens* but are noticeably longer, thus making them appear more slender. On *A. tenerum* the hairs are finer, closer together, and more acutely pointed than in the case of the two others.

SUMMARY

The individual seeds of *Agropyron repens*, *A. smithii*, and *A. tenerum*, with the exception of some terminal or immature seeds, can be identified as to species by a study of the characters, such as form, color, texture, concavity of palea, outline of palea tip, the shape of the rachilla segment, and the width of the opening between the edges of the lemma at the base of the rachilla.

In identifying seeds of quack grass (*Agropyron repens*) the shape of the rachilla and the width of the opening between the edges of the lemma at the base of the rachilla segment are valuable diagnostic characters.

Familiarity with the extremes in form observed in occasional seeds of a species but not duplicated in either of the other two species enables the analyst to identify seeds of these types at sight.

With the combined study of the differences of general form and the differences in quality and quantity of pubescence, all individual seeds of *Agropyron repens*, *A. smithii*, and *A. tenerum* should be easily identified.

[7] The writer evidently means dorsal instead of ventral. Terms interchanged also in following sentence.

THE TOXIC CONSTITUENT OF RICHWEED OR WHITE SNAKEROOT (EUPATORIUM URTICAEFOLIUM)[1]

By JAMES FITTON COUCH

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HISTORICAL REVIEW

Among the many perils and hardships that marked the settlement of the old frontier of the United States was the mysterious malady known as "milk sickness." Appearing customarily late in the summer it rapidly assumed epidemic proportions, sparing neither age nor sex, frequently sweeping away a quarter of the population, and sometimes depopulating whole villages. Nearly every district in the old West—now the Central West—suffered at one time or another from this disease and so sudden was its onset, so recondite its cause, so unavailing were the preventive measures taken by the terrified backwoodsmen, and so fatal were the attacks, that the old chroniclers refer to it in the strongest terms as formidable, terrible, and frightful. Nicolay and Hay (*17*)[2] describe a characteristic outbreak during which the mother of Abraham Lincoln died.

> In the autumn of 1818 the little community of Pigeon Creek was almost exterminated by a frightful pestilence called the milk sickness, or, in the dialect of the country, "the milk sick." It is a mysterious disease . . . It seems to have been a malignant form of fever—attributed variously to malaria and to the eating of poisonous herbs by the cattle—attacking cattle as well as human beings, attended with violent retching and a burning sensation in the stomach, often terminating fatally on the third day. In many cases those who apparently recovered lingered for years with health seriously impaired.

The disease was known in Maryland, North Carolina, Kentucky, Tennessee, Alabama, Missouri, Illinois, Indiana, and Ohio from the time of the early settlements. Wolf, Curtis, and Kaupp (*22*) in one of the most complete accounts of this sickness that has ever been published, have shown that milk sickness was recognized as a particular disease in North Carolina before the Revolution. The early settlers were also acquainted with a disease of cattle known as "trembles" that appeared only in "milk-sick areas" and during the same periods in which human milk sickness was prevalent. It was observed, too, that suckling calves frequently sickened and died from "trembles" in these same milk-sick areas and that small domestic animals, pigs, cats, and dogs, fed on the milk of cows that were permitted to range the woods, developed symptoms of nausea, weakness, and enteritis, and died. These facts led to the suspicion that the milk of cows at certain seasons contained some deleterious substance that was capable of causing milk sickness in human beings who drank it or ate butter and cheese made from it. This idea was further strengthened by the fact that those who prudently abstained from all bovine products late in summer and fall were free from attacks of

[1] Received for publication May 12, 1927; issued November, 1927.

[2] Reference is made by number (italic) to "Literature cited," p. 575.

milk sickness and that those who discontinued eating milk products as soon as they began to sicken with the disease generally recovered after suffering merely a slight illness.

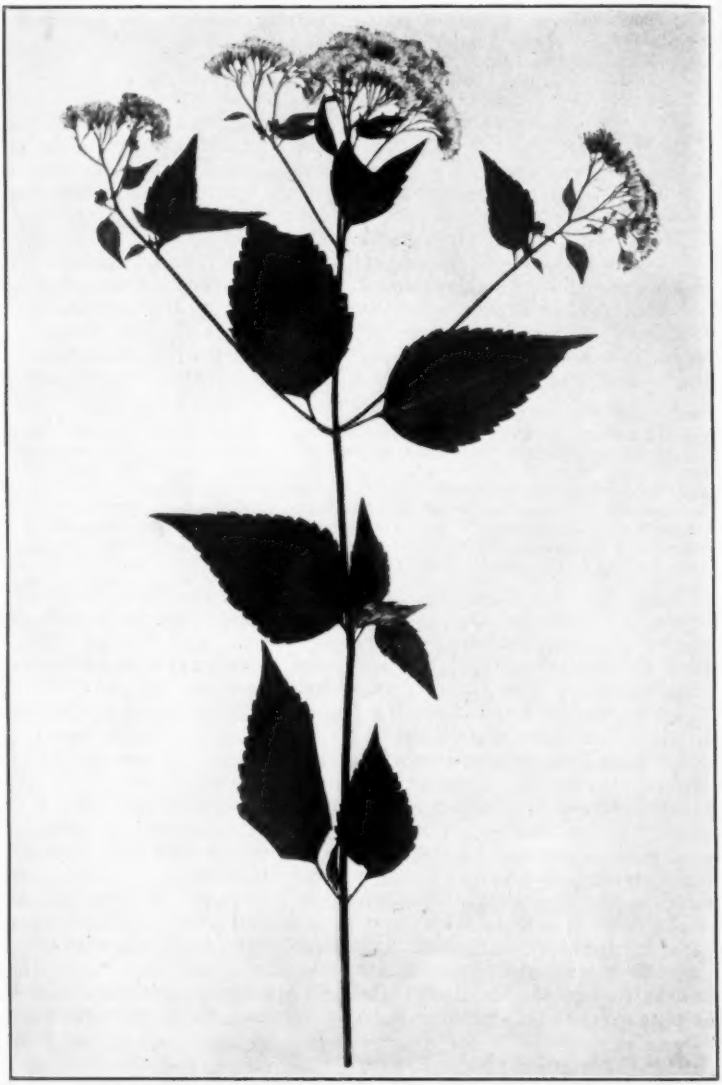

FIG. 1.—Richweed or white snakeroot (*Eupatorium urticaefolium*)

The nature of the deleterious substance present in this poisonous milk remained obscure. Speculation as to its character was widespread and resulted in the advancement of many fantastic ideas.

Poisonous dew and volatile minerals that evaporated from the earth at night, condensing on the herbage, there to await ingestion by the grazing herds, miasmata, the early conception of a germ theory, microorganisms, various poisonous minerals, arsenic, copper, lead, cobalt, as well as poisonous springs, all were advanced as explanations of the origin of the scourge. By far the most favored theory, however, and one held tenaciously from the earliest times was that milk sickness was caused by the feeding of the cows on some weed or herb that could transmit poisons to the milk. This hypothesis was strengthened by a large number of observed facts. The disease was most virulent late in summer and early fall, particularly after a period of drought, when wholesome forage plants had been consumed and the cattle were compelled to graze on plants that they refused when other feed was plentiful. Again, cattle that were pastured in inclosed fields where weed infestation was slight did not contract the trembles nor did their milk cause milk sickness. It was observed that the incidence of milk sickness diminished as the forests were cleared and the land brought under cultivation. Many different plants were suspected to be the cause. Some of them, such as poison ivy, water hemlock (*Cicuta maculata*), "Indian hachy," Indian tobacco (*Lobelia inflata*), Indian hemp (*Apocynum cannabinum*), Virginia creeper (*Parthenocissus quinquefolia*), cross vine (*Bignonia capriolata*), Indian currant (*Symphoricarpos orbiculatus*), marsh marigold (*Caltha palustris*), spurge (*Euphorbia esula*), fool's parsley (*Arethusa cynapium*), mushrooms, and richweed (*Eupatorium urticaefolium*) were well known as poisonous plants. Others argued in favor of the modified hypothesis that the disease was caused by parasitic fungi or by molds that grew on various plants.

The last-named plant, *Eupatorium urticaefolium*, known commonly as richweed, white snakeroot, pool wort, pool root, white sanicle, Indian sanicle, deer wort, squaw weed, white top, and steria, has been most widely and commonly held responsible for the disease. A great many experiments have been made in which this plant has been fed to animals with the result that trembles has been produced in a large number of cases. In recent years a mass of proof has been obtained to show that the plant is poisonous to livestock and that suckling animals may be poisoned by the milk secreted by animals that have eaten it.

Notwithstanding the many evidences of the poisonous nature of richweed and the many indications of its connection with human milk sickness, some observers have doubted its relation either to trembles or to milk sickness, and have advanced very strong arguments against the conclusion. They have pointed out that cattle have been known to graze for long periods in places where richweed grows abundantly without the appearance of any cases of trembles in the cattle or of milk sickness in the people who drank their milk. They further contended that in many of the feeding experiments the animals failed to develop abnormal symptoms. Finally, it was discovered that a disease resembling trembles in cattle and milk sickness in human beings exists in certain places in New Mexico and Texas where richweed is not found.

It is quite true that cattle have been known to graze with impunity in pastures infested with richweed, but it is also true that stock do not voluntarily eat this plant so long as other forage is available;

and the mere presence of richweed in a pasture does not prove that it has been eaten by the animals confined there.

Failure to produce the disease by feeding the plant is inconclusive evidence unless supported by a thorough and systematic course of experiments. One feeding of *Eupatorium urticaefolium*, unless in very large quantity, would certainly fail to provoke symptoms of trembles, and the result of such a feeding may lead to an uncritical opinion that the plant is harmless. Feeding of dried or partly dried plant would also fail to produce the disease in many if not in all cases. Furthermore, it is by no means certain that there is not a variation in the toxicity of richweed at different seasons or in different years or in plants from different localities.

The discovery of endemic milk sickness in the Pecos Valley of New Mexico, where *Eupatorium urticaefolium* does not grow, was considered strong evidence against the conclusion that richweed caused that disease. It has been shown, however, by a number of investigators that milk sickness and trembles in New Mexico are caused by the rayless goldenrod, *Aplopappus heterophyllus* (A. Gray) (Blake), also termed *Isocoma wrightii, Isocoma heterophylla, Bigelovia rusbyi*, and *Bigelovia wrightii*.

The theory that milk sickness is due to some specific microorganism has been held by several observers (*11*). Jordan and Harris (*9*), in a very careful and detailed examination of the possibility of pathogenic microorganisms being the cause of the disease, isolated a bacillus that appeared to be a possible solution to the problem. This organism they named *Bacillus lactimorbi* and they made an extensive study of it. At the end of their research, however, they candidly stated (*9, p. 484–485*):

"Taken as a whole the facts do not surely indicate that a specific microorganism is the cause of milk sickness or trembles."

The work of Jordan and Harris was supplemented by a bacteriological study of their organism by Luckhardt (*10*) in which various strains of the organism fed by him to dogs did not produce typical trembles. In pathogenicity the organism was very erratic. Luckhardt found it very widespread and growing on plants, such as alfalfa, that have never been associated with milk sickness. His study was, as he says, "far from being decisive in establishing *B. lactimorbi* as the etiological factor in the production of milk-sickness."

At the present time the only hypothesis that has any standing is that richweed poisoning causes this disease in the East. All the experimental work reported in the last decade supports this point of view and demonstrates that *Eupatorium urticaefolium* is a distinctly poisonous plant and that milk obtained from cows that have fed on it is capable of producing the disease in other animals.

Full descriptions of the history and characteristics of the disease have been published (*11, 8, 2, 18, 22*).

Apparently the first published account of milk sickness was included by Daniel Drake (*4*), who published the information concerning the disease that Barbee, of Virginia, collected while visiting the Mad River country of Ohio, in 1809, and other data gathered by Drake himself. In a larger work (*5*) Drake again notices the milk sickness and mentions the fact that some ascribe it to the poison of some unknown plant that is secreted in cow's milk. Meanwhile Hall

(*6*) had published the case of Alex. Telford and family in which the milk from four cows was strongly suspected of causing the outbreak.

A great deal has been written about milk sickness in the medical journals and considerable controversial material has developed in which even the existence of the disease as a specific malady has been denied. Some cursory experiments were made to determine the toxic properties of richweed but they were often of such a nature as to yield little positive knowledge.

About 20 years ago Moseley (*13*) reported a series of experiments with laboratory animals that indicated toxic properties in richweed. Crawford (*2*) reviewed the work, made some experiments, and concluded that there is no relationship between richweed and milk sickness. The distinctly poisonous nature of the plant, however, was demonstrated beyond question by a series of researches published during the last 10 years by Curtis and Wolf (*3*), who showed that the disease was not infectious; by Marsh and Clawson (*12*); Wolf, Curtis, and Kaupp (*22*), who poisoned animals on the sap of the plant; and by Sackett (*19*), who was able to poison laboratory animals with extracts of the plants. The results of Sackett's experiments further confirmed the toxic character of richweed.

When the writer began the study of this subject in 1918, the principal problem to be solved was the isolation of the toxic constituent and the elucidation of its chemical properties.

PREVIOUS CHEMICAL INVESTIGATIONS OF RICHWEED

No complete chemical analysis has yet been made of the whole plant of *Eupatorium urticaefolium*, although a number of investigators in the course of studies of the toxic constituent, have made extracts of the plant and have fed these to animals.

Moseley (*13*) fed extracts of the plant in water and milk to rabbits and dogs, and also gave parts of the plant to these animals. Crawford (*2*) injected aqueous extracts subcutaneously into two rabbits which died and into three others which survived. These extracts were made from dried plants. Extracts from plants preserved in chloroform water failed to injure a cat, a dog, a sheep, and Crawford himself, when fed in small dosage. Injection of the ash, obtained from 10 and 20 gm. of dried plant and neutralized with acetic acid, into rabbits, did not produce poisoning.

In 1909 Moseley (*14*) published his conclusions that the toxic constituent of richweed is aluminum phosphate, and adduced experimental evidence in support of this hypothesis. In 1910 Moseley (*15*) published further evidence to support the aluminum phosphate hypothesis, and advocated the use of soda as an antidote.

Curtis and Wolf (*3*) fed aluminum phosphate in doses of from 2 to 16 gm. to two ewes during a period of 69 days, at the expiration of which each had received 412 gm. of the compound. Neither of the ewes developed any abnormal symptoms and each gained weight.

Mosely (*16*) withdrew the aluminum phosphate hypothesis in favor of the conclusion that an "ether soluble resin" is the toxic principle. He reported that he obtained 9 per cent of the dry weight of the leaves by extracting with ether and that nearly all the extract

was resin. He does not record his experiments but states that the resin poisons cats, rabbits, and guinea pigs like Eupatorium.

Wolf, Curtis, and Kaupp (*22*) in an excellent monograph describe the most extensive use of extracts that had appeared up to that time. Aqueous and alcoholic extracts fed by them to guinea pigs did not produce poisoning. One of three guinea pigs to which they fed hydrochloric acid extract died. The expressed juice of fresh plant was fed to five guinea pigs, four of which died. Dried extract from the sap prepared by exsiccation on a water bath at 100° C. did not affect three guinea pigs to which it was fed. The volatile matters from the sap fed to three guinea pigs did not affect the animals. The inspissated juice evaporated at a temperature less than 60° C. fed to three guinea pigs caused the death of one. The other two merely showed some depression. Five sheep were fed the expressed juice of richweed and died with symptoms of trembles; two sheep were fed inspissated juice evaporated at a temperature below 60° C. and died with symptoms of trembles; and a sheep was fed the nonvolatile portions of the juice and developed trembles and died. These investigators were able to produce trembles in two out of six suckling lambs by feeding richweed to the mothers. A sample of "milk-sick" butter was fed to three mice, two of which died. Seven dogs were fed meat from trembles cases and showed no ill effects. As a result of these experiments the investigators concluded that the toxic constituent of richweed is glucosidal. In all their experiments they used green plant collected at Shooting Creek, N. C.

A valuable contribution to the subject was made by Sackett (*19*), who conducted many experiments with extracts from the leaves of *Eupatorium urticaefolium* on rabbits and guinea pigs. Fresh and carefully dried leaves and alcohol and chloroform ether-ammonia (Prolius's solution) extracts of richweed fed by him produced poisoning in animals. The alcohol and Prolius's solution marcs were nontoxic as were the extracts made with salt solution and that from the roots. Viscera and meat from a rabbit killed by richweed poisoning did not affect a cat. An anaphylactic experiment was negative. Guinea pigs were not affected by the dried leaves.

Jordan, Whelan, and Gidley (*7*) made some experiments with extracts from dried plants collected in Indiana. These were fed to rabbits and the marc from a 0.1 per cent hydrochloric acid extraction was fed to a hen. The 50, 70, and 95 per cent alcohol extracts, an ether-chloroform extract, and the extract made with a menstruum of 50 per cent alcohol and 0.5 hydrochloric acid were nontoxic. A 0.5 per cent hydrocloric acid extract killed a rabbit in four hours. A 0.1 per cent hydrochloric acid extract was fed to a rabbit which died in three days; the marc from this fed to a hen produced no effect. A similar 0.1 per cent hydrochloric acid extract which had fermented produced no result when fed to a rabbit. The resin was nontoxic.

These investigators report that there is neither alkaloid nor glucoside in the plant.

Bukey (*1*) reported a partial analysis of the fruit of *Eupatorium urticaefolium*, which includes data concerning a drying oil that he extracted, together with a proximate analysis of the other constituents.

From this contradictory mass of data it may be concluded that: The toxic principle appears to be soluble in alcohol, ether, and chloroform, and insoluble in water; it is apparently nonvolatile and is destroyed or greatly weakened by drying the plant; it appears to be thermostable within reasonable limits; nothing is certain about its chemical characters.

THE PRESENT INVESTIGATION

The result of the present investigation has been the isolation of a definite, toxic substance from green *Eupatorium urticaefolium* which appears capable of producing the characteristic symptoms of trembles in sheep and of a second toxic substance, not yet obtained in a pure condition, which is toxic to guinea pigs but does not affect sheep even in very large doses and probably has no relationship to milk sickness.

TREMETOL

The substance which appears to be responsible for trembles is a viscous, oily liquid of pleasant, aromatic odor which does not solidify at very low temperatures and can not be made to crystallize. Analysis and molecular-weight determinations indicate that the formula of this substance is $C_{16}H_{22}O_3$. It has been named tremetol from tremere, to tremble, inasmuch as all feasible names derived from the generic name of the plant have already been used to designate other substances. Tremetol has the characters of an alcohol; the hydroxyls are not phenolic and alkoxyl groups appear to be absent. It does not react with hydroxylamine or with phenylhydrazine to form crystalline substances, nor does it give color reactions with Schiff's reagent or with ferric chloride. It withstands the temperature of boiling water but is rapidly decomposed when attempts to distill it are made, even at pressures below 1 mm. It is very slowly volatile with steam. It is soluble in petroleum ether, chloroform, alcohol, and benzene, and quite insoluble in water, acids, or alkaline solutions. It is levorotatory in alcoholic solution, $[\alpha]_D^{20} = -21.08°$. It absorbs four atoms of bromine, which indicates the presence of two double bonds.

Potassium permanganate oxidizes it to a crystalline acid that melts at 181° C. Tremetol apparently exists in the plant partly in ester combination with a resinous acid, the chemistry of which is still being studied. The toxic substance may be prepared by the following method.

Method of Preparing Tremetol

To obtain tremetol the fresh richweed plant is hashed through a meat chopper and immediately packed in a percolator with strong alcohol, macerated two days and then percolated to exhaustion with strong alcohol. The solvent is distilled from the percolate at ordinary pressure, leaving a watery residue mixed with a quantity of resin and fat. This is repeatedly extracted with boiling water to remove all water-soluble constituents. The water layers are siphoned off after each extraction. The final resinous residue is then extracted with boiling 50 per cent alcohol as long as soluble matter is obtained.

The alcoholic solutions are filtered off hot, united, and distilled to remove alcohol. The watery residue is allowed to cool and is filtered from precipitated resins. These resins are then extracted with boiling 30 per cent alcohol as long as they yield soluble matter. The solutions are filtered hot and the alcohol is removed from them. Crude tremetol ester separates out. This is purified by solution in alcohol and by boiling with 5 per cent alcoholic potash to hydrolyze the ester.

When hydrolysis is complete the alcohol is evaporated from the reaction product, and the residue is dissolved in water and extracted with ether, which dissolves the tremetol together with some coloring matters. The ether solution is poured into four volumes of petroleum ether, as a result of which process most of the coloring matters and other impurities are precipitated. Tremetol is recovered by evaporating the solvent, and should be purified by repeated solution in ether and precipitation with petroleum ether. The purity of the product may be determined by optical rotation and by ascertaining molecular weight. The absence of ash and deep color also indicates purity.

A Color Reaction of Tremetol

When a small quantity of tremetol is dissolved in petroleum ether and the solution is floated on the surface of concentrated sulphuric acid contained in a test tube, a red ring forms at the interface. If the two liquids are mixed by gentle agitation the petroleum ether is colored a transient cherry red which rapidly fades and the acid layer acquires a brilliant cherry-red color. If the solution is too dilute a yellow or orange may be the only result; if it is too concentrated or if much impurity is present the color reaction may be obscured by carbonization.

This reaction is offered as a provisional test for the presence of tremetol in Eupatorium mixtures. While there are numerous other substances which yield red colors with concentrated sulphuric acid, many of them are insoluble in petroleum ether and would not interfere with the test. Others give different shades of red. The cherry red given by tremetol is quite characteristic. A negative test definitely establishes the absence of tremetol; a positive reaction taken in conjunction with other data may demonstrate the presence of that substance.

A careful study of the applicability and specific nature of this test is in progress. It is hoped that through this color reaction a method may be developed by which suspected milk may be tested and dangerous milk detected, in areas where milk sickness exists.

It has been possible to use this test in order to check up on feeding experiments, particularly when failure to produce poisoning by extracts was not explicable. Unfortunately, samples of some of the extracts used during the course of this study were not available when the test was discovered and could not be tested. The results are stated in connection with the various discussions of the extracts.

COLLECTION OF PLANT MATERIAL

The plant material used in this investigation was collected in two localities in Illinois and in one place in Virginia, as shown in Table 1. The Illinois plant was gathered partly in the neighborhood of Beecher

City and partly at Paxton, in both of which localities milk sickness had appeared. The Virginia plant was collected on the banks of the Potomac River, Arlington County, in the neighborhood of Washington, D. C., a district from which no record of milk sickness has come. Both green and dried plants were studied. The dried plant originated wholly in Beecher City, Ill. One lot of green plant was collected at Beecher City, and was packed in milk cans, covered with chloroform water to preserve it, and shipped to Washington, D. C. Other lots of green material from Illinois were mailed to Washington where they arrived in fresh condition and were immediately hashed and put into strong alcohol. Green material was collected in Virginia and was carried to the laboratory within 24 hours. When used for direct feeding, the plant was usually collected early in the morning and was carried immediately to the laboratory. One lot of green plant, collected in Virginia by W. N. Berg, was immediately placed by him in strong alcohol and had been macerated for two years when it was worked up during the present study.[3]

TABLE 1.—*Collections of Eupatorium urticaefolium*

Lot No.	Date collected	Condition when used	Place of collection	Weight	Collector
				Kgm.	
E 1	Nov. 17, 1916	Green	Arlington Co., Va	1.805	W. N. Berg.
E 2	October, 1915	Dry	Fancher (Beecher City), Ill.	4.432	A. B. Clawson.
E 3	Oct. 7-22, 1921	Green	Woodmont, Va	13.63	J. F. Couch.
E 4	Apr. 20-30, 1925	do	do	7.27	Do.
E 5	Sept. 22, 1920	do	Beecher City, Ill	28.0	Do.
E 6	Sept. 25-Oct. 3, 1921	do	Woodmont, Va	53.32	Do.
E 7	October, 1922	do	Beecher City and Paxton, Ill.	35.9	J. M. Casstevens and J. E. Parks.
E 8	October, 1915	Dry	Beecher City, Ill	18.93	A. B. Clawson.
E 9	October, 1923	Green	Beecher City and Paxton, Ill.	98.5	J. M. Casstevens and J. E. Parks.
E 10	September, 1925	do	Beecher City, Ill	131.8	J. M. Casstevens.

ANIMALS USED IN THE EXPERIMENTAL WORK

The earlier experiments to test the toxicity of various extracts and their fractions were made on guinea pigs, but the variability of the results obtained with these animals and the impossibility of their exhibiting the symptoms characteristic of trembles in cattle led to abandonment of guinea pigs in favor of sheep. It was on the latter animal that all the crucial experiments were made. Cats were used in certain feeding tests, and rabbits were used in testing the toxicity of the resin acid.

All animals were allowed access to plenty of feed and water during the experiments. These experiments are summarized in Table 2.

[3] The writer desires to express his thanks to those who assisted in the collection of plant material, and especially to J. M. Casstevens and to J. E. Parks, of Illinois. Specimens of the various collections were submitted to S. F. Blake, botanist at the Bureau of Plant Industry, for identification before they were used in this investigation.

TABLE 2.—*Summary of experiments in feeding Eupatorium extracts to animals*

Date of feeding		Animal		Material fed			Dosage		Plant equivalent		Character of constituents	Effect	Termination
On or from—	To—	No.	Weight	Condition of plant	Origin	Fraction and laboratory No.	Quantity per single dose	Number of doses	Per single dose	Total			
1919	1919	*Sheep*	*Pounds*										
July 1		539	96	Dry	Fancher, Ill.	E 2. ABC	48.75 c. c.	4	3 lb.	12 lb.	Part of water solution.	None	Survived.
2		539	96	do	do	E 2. ABC	73 c. c.	4	4.5 lb.	18 lb.	do	do	Do.
5	July 8	550	87	do	do	E 2. AAA	1 gm	12	150 gm.	1,800 gm.	Resin acids	do	Do.
8		550	87	do	do	E 2. AAA	2 gm	4	300 gm.	1,200 gm.	do	do	Do.
9	July 10	550	83.25	do	do	E 2. AAA	3 gm	7	450 gm.	3,150 gm.	do	do	Do.
11		550	83.25	do	do	E 2. AAA	4 gm	4	600 gm.	2,400 gm.	do	do	Do.
12		550	81	do	do	E 2. AAA	5 gm	4	750 gm.	3,000 gm.	do	do	Do.
13		550	83.75	do	do	E 2. AAA	15 gm	2	2,250 gm.	4,500 gm.	do	do	Do.
15		522	86	do	do	E 2. AAB	150 c. c.	1	1,456 gm.		Lipoid fraction	do	Do.
16		522	86	do	do	E 2. AAB	150 c. c.	1	2,912 gm.		do	do	Do.
Aug. 19		534	108.5	do	do	E 2. alkaloid		1	24.4 lb.		Alkaloid	do	Do.
26	Sept. 2	550	98	do	do	E 2. marc	214 gm	4	214 gm.	856 gm.	Marc	do	Do.
1920													
June 28		596	94.5	do	do	E 2. B	60 c. c.	1	3 lb.		Alcohol-insoluble, water solution.	do	Do.
29	June 30	596	94.5	do	do	E 2. B	100 c. c.	3	5 lb.	15 lb.	do	do	Do.
July 1		596	94.5	do	do	E 2. B	150 c. c.	1	7½ lb.		do	do	Do.
2		596	94.5	do	do	E 2. B	200 c. c.	2	10 lb.	20 lb.	do	do	Do.
1921													
July 6	July 19	639	86.5	Green	Beecher City, Ill.	E 5. BAAA	381 c. c.	14	2.5 lb.[a]	35 lb.[a]	Nonacidic, volatile	do	Do.
16	July 31	650	72	do	do	E 5. BAB	162 c. c.	16	2.5 lb.[a]	40 lb.[a]	Nonvolatile, water soluble.	do	Do.
22	Aug. 4	622	85	do	do	E 5. AAA	50 c. c.	14	2.5 lb.[a]	35 lb.[a]	Resin acids	Pulse depressed	Do.
Aug. 2	Aug. 8	631	88	do	do	E 5. BAAB	3.45 gm	7	2.5 lb.[a]	17.5 lb.[a]	Volatile acids	None	Do.
30	Sept. 5	625	85.5	do	do	E 5. AAB	65 c. c.	7	2.5 lb.[a]	17.5 lb.	Lipoid fraction	Trembles	Recovered.
Sept. 6	Sept. 13	650	88	do	do	E 5. AAB	66 c. c.	7	2.5 lb.[a]	17.5 lb.[a]	do	do	Killed for autopsy.
1922													
July 10	July 21	677	98.75	do	Woodmont, Va.	E 6.3 AB	8.7 gm	14	2.5 lb.[a]	35 lb.[a]	Ligroin solution of lipoid fraction.	Fever	Survived.
11	July 22	679	78.5	do	do	E 6.5 AC	0.872 gm	12	2.5 lb.[a]	30 lb.[a]	Alcohol fraction of fats and resins.	None	Do.
15	July 25	674	73	do	do	E 6.4. AB	3.63 gm	18	2.5 lb.[a]	40 lb.[a]	Benzene solution of lipoids.	do	Do.

24	...do	679	78.5	...do	...do	E 6.5 AC	1.75 gm	2	5 lb.*	10 lb.*	Alcohol fraction of fats and resins.	...do	Do.
24	...do	674	73	...do	...do	E 6.4 AB	7.26 gm	2	5 lb.*	10 lb.*	Benzene solution of lipoids.	...do	Do.
27	Aug. 1	679	78.5	...do	...do	E 6.5. AC and 4 AB.	13.3 gm	6	2.5 lb.*	15 lb.*	Mixture of ligroin and benzene fraction.	...do	Do.
1923													
Mar. 13	Mar. 16	707	62	...do	Beecher City, Ill.	E 7.3 A	25.9 gm	4	4.6 lbs.*	18.4 lb.*	Fats and resin acids.	Trembles	Died Mar. 18.
Apr. 3	Apr. 11	708	82.5	...do	...do	E 7.3 AB	24.3 gm	4	4 lb.*	16 lb.*	Fatty acids	None	Survived.
16	Apr. 18	708	67	...do	...do	E 7.4 A	10.22 gm	3	4 lb.*	12 lb.*	Sterols	Trembles	Died.
16	Apr. 22	709		...do	...do	Milk of 708						...do	Killed for autopsy.
July 3	July 7	779	38	...do	...do	E 7.4 AB	1.2 gm	4	4 lb.*	16 lb.*	Phytosterol fraction.	None	Survived.
11	July 14	779	38	...do	...do	E 7.6 AII	1 gm	4			Vacuum distillate No. 2.	...do	Do.
20	July 21	779	38	...do	...do	E 7.6 AII	1.5 gm	2			...do	...do	Do.
25	July 28	779	38	...do	...do	E 7.6 A 4	2 gm	4	5 lb	20 lb	Vacuum residue	...do	Do.
Aug. 2	Aug. 6	779	38	...do	...do	E 7.5 AB	1 gm	4			Ligroin insoluble	...do	Do.
14		779	38	...do	...do	E 7.5 AB	3 gm	1			...do	...do	Do.
20	Aug. 21	779	38	...do	...do	E 7.5 AII	1.5 gm	2			Vacuum distillate No. 3.	...do	Do.
20	Aug. 23	779	38	...do	...do	E 7.6 AIII	0.46 gm	4			...do	...do	Do.
28	Aug. 31	779	38	...do	...do	E 7.4 AB	2.5 gm	4	8 lb.*	32 lb.*	Phytosterol fraction.	...do	Do.
Sept. 1		779	38	...do	...do	E 7.4 AB	2 gm	1	6.4 lb.*		...do	...do	Do.
4	Sept. 5	779	38	...do	...do	E 7.4 AB	2.5 gm	2	8 lb.*	16 lb.*	...do	...do	Do.
12	Sept. 13	779	38	...do	...do	E 7.5 A	3.5 gm	2	15.67 lb.*	31.34 lb.*	Ligroin soluble	Symptoms	Recovered.
17	Sept. 19	779	38	...do	...do	E 7.6 AI	4.26 gm	3			Vacuum distillate No. 1.	None	Survived.
25		779	38	...do	...do	E 7	5 gm	1	12.6 lb	33.1 lb. *	Volatile oil	Not observed	Died.
1924	1924												
Jan. 2	Jan. 3	782	79	Dry	...do	E 8. B	1.25 gm	4			Vacuum distillates	None	Survived.
Apr. 15	Apr. 30	782	105	Green	...do	E 7	1 gm	13	2.52 lb	32.75 lbs*	Volatile oil	...do	Do.
June 9	June 12	782	105	...do	...do	E 9. AAA	2 gm	4	4.4 lb.*	17.6 lb.*	Alcohol-insoluble oil from sterol fraction.	...do	Do.
18	June 21	782	105	...do	...do	E 9. AAC	0.5 gm	4			Steam distillate	...do	Do.
24	June 25	782	105	...do	...do	E 9. AAD	2.9 gm	2	5.5 lb	11 lb	Sterol residue from steam distillate.	...do	Do.
26	June 27	782	105	...do	...do	E 9. AAD	2.3 gm	2	4.4 lb	8.8 lb	...do	...do	Do.
30	July 5	782	105	...do	...do	E 9. AAD	4.6 gm	5	8.8 lb	44 lb	...do	...do	Do.
July 15	July 18	782	105	...do	...do	E 9. AAA	3.75 gm	4	7.15 lb	28.6 lb	Oil from sterol fraction.	...do	Do.

* Per hundredweight of animal.

TABLE 2.—*Summary of experiments in feeding Eupatorium extracts to animals*—Continued

Date of feeding		Animal		Material fed			Dosage		Plant equivalent		Character of constituents	Effect	Termination
On or from—	To—	No.	Weight	Condition of plant	Origin	Fraction and laboratory No.	Quantity per single dose	Number of doses	Per single dose	Total			
1925	1925	*Sheep*	*Pounds*										
Feb. 9	Feb. 12	782	.....	Dry	Beecher City, Ill.	E 9. A	27.4 gm	4	4 lb.[a]	16 lb.[a]	Alcohol extract	None	Survived.
Apr. 1	Apr. 9	4	57	do	do	E 9. A	15 gm	8	4 lb.[a]	32 lb.[a]	do	do	Do.
22	Apr. 25	4	60	do	do	E 9. A	30 gm	4	8 lb.[a]	32 lb.[a]	do	do	Do.
June 20	June 30	5	75	do	Woodmont, Va.	E 4	1.66 lb.[a]	9	1.66 lb.[a]	15 lb.[a]	Green plant	do	Do.
1926	1926												
Mar. 4	Mar. 6	5	128	do	Beecher City, Ill.	E 10. AAB	7 gm	3	1.65 lb.[a]	5 lb.[a]	Solution in 50 per cent alcohol.	do	Do.
7	Mar. 18	5	128	do	do	E 10. AAB	10.56 gm	9	2.5 lb.[a]	22.5 lb.[a]	do	do	Do.
22	Apr. 3	5	123	do	do	E 10. AAA	27 gm	12	3 lb.[a]	36 lb.[a]	Insoluble in 50 per cent alcohol.	do	Do.
Apr. 12	Apr. 15	5	123	do	do	E 10. AAB	21.12 gm	4	5 lb.[a]	20 lb.[a]	Soluble in 50 per cent alcohol.	Trembles	Died.
May 8	May 13	6	69	do	do	E 10. AABB		5	7.5 lb.[a]	37.5 lb.[a]	Insoluble in 30 per cent alcohol.	None	Survived.
Aug. 3	Aug. 10	6	70	do	do	E 7. AAB	1.95 gm	7	7.5 lb.[a]	52.5 lb.[a]	Resin acid	do	Do.
20	Aug. 25	6	75	do	do	E 7. B	100 gm	5	8 lb.[a]	40 lb.[a]	Water soluble	do	Do.
1918	1918	*Guinea pig*	*Grams*										
Nov. 20	.....	52	375	Green	Arlington County, Va.	E 1. AB	0.08 gm	10	10 gm	100 gm	Volatile oils and fats	None	Do.
20	.....	53	365	do	do	E 1. AB	0.08 gm	10	10 gm	100 gm	do	do	Do.
21	Nov. 27	46	560	do	do	E 1. ACB	13.13 gm	5	65.6 gm	328.2 gm	Water soluble	do	Killed for autopsy.
29	.....	55	305	do	do	E 1. AA		1	900 gm	.....	Resin	Sick	Died Nov. 30.
Dec. 13	Dec. 19	57	310	do	do	E 1. AAB		6	150 gm	900 gm	Soda solution of resin	do	Died Dec. 31.
1919	1919												
Jan. 2	.....	70	535	do	do	E 1. AAA		1	900 gm	.....	Resin insoluble in soda.	do	Died Jan. 15.
1921	1921												
Oct. 7	Oct. 22	192	670	do	Woodmont, Va.	E 3. fresh leaves.		10	21.75 gm	217.5 gm	Total	do	Survived.
7	do	193	590	do	do	do		10	21.75 gm	217.5 gm	do	do	Died Nov. 9.
Dec. 13	Dec. 19	197	560	do	do	E 6.3 AB	1 gm	3	130 gm	390 gm	Ligroin solution	do	Recovered.

1922 Jan. 26	1922 Feb. 16	199	490	do	do	E 6.4 AB	0.3 gm	14	78.3 gm	1,096.2 gm	Benzene solution	None	Survived.
26	do	200	590	do	do	E 6.4 AB	0.3 gm	14	78.3 gm	1,096.2 gm	do	do	Do.
Feb. 20	Feb. 28	201	550	do	do	E 6.5 AB	0.1 gm	7	200.7 gm	1,405 gm	Ether solution	do	Do.
20	do	202	485	do	do	E 6.5 AB	0.1 gm	7	200.7 gm	1,405 gm	do	do	Do.
Mar. 15	Mar. 20	201	550	do	do	E 6. 7 A	0.2 (1 of 0.15)	4		1,405 gm	Alcohol soluble resin acids.	Sick	Died Apr. 20.
15	do	202	485	do	do	E 6.7 A	0.2 (1 of 0.15)	4		1,405 gm	do	None	Survived.
8	Apr. 1	199	635	do	do	E 6.3 AB	0.381 gm	21	49.6 gm	1,041.6 gm	Ligroin solution	do	Do.
8	do	200	715	do	do	E 6.3 AB	0.381 gm	21	49.6 gm	1,041.6 gm	do	Sick	Died Apr. 9.
1923 Feb. 28	1923 Mar. 1	213	490	do	Beecher City, Ill.	E 7.3 A	0.2 cc	2	10 gm	20 gm	Fats, resin, and resin acids.	do	Died Mar. 23.
June 14		236	650	do	do	E 7.6 A II	0.2 cc	1			Vacuum fraction No. No. 2.	do	Recovered.
14		211	725	do	do	E 7.6 A II	0.3 cc	1			do	do	Do.
14		239	700	do	do	E 7.6 A II	0.5 cc	1			do	do	Died.
July 8	1926	293	485	do	do	E 10	0.2 c. c.	1			Tremetol	do	Survived.
8		294	420	do	do	E 10	0.1 c. c.	1			do	do	Died.
8		295	410	do	do	E 10	0.15 c. c.	1			do	do	Do.
8		296	445	do	do	E 10	0.3 c. c.	1			do	do	Survived.
27		293	485	do	do	E 10	0.25 c. c.	1			do	do	Died.
27		296	445	do	do	E 10	0.3 c. c.	1			do	do	Do.
Aug. 9	Aug. 20	297	460	do	do	E 7. AAB	1.052 gm	3	63.46 gm	190.46 gm	Resin acid	do	Do.
9	11	298	430	do	do	E 7. AAB	0.078 gm	2	95.24 gm	190.46 gm	do	do	Do.
9	Sept. 2	299	545	do	do	E 7. AAB	0.218 gm	5	266.6 gm	1,333 gm	do	do	Recovered.
9	do	300	470	do	do	E 7. AAB	0.097 gm	5	114.25 gm	571.28 gm	do	do	Do.
9	Aug. 20	301	510	do	do	E 7. AAB	0.156 gm	3	190.46 gm	571.36 gm	do	do	Died.
9		302	560	do	do	E 7. AAB	0.156 gm	1	190.46 gm	190.46 gm	do	do	Do.
1923 Mar. 19	1923 Mar. 28	*Cat* 17	1,680	(b)					711 gm		Liver, spleen, heart, kidney.	None	Survived.
19	do	18	1,630	(b)					1.450 gm		Muscle tissue	do	Do.
19	do	19	1,530	(b)					1.600 gm		do	do	Do.
Nov. 26		17	2,000	Green	Beecher City, Ill.	E 7. oil	0.45 gm	1			Essential oil	do	Do.
26		19	2,840	do	do	E 7. oil	0.30 gm	1			do	do	Do.
27		17	2,000	do	do	E 7. oil	0.60 gm	1			do	do	Do.
27		19	2,840	do	do	E 7. oil	0.60 gm	1			do	do	Do.
1926 Aug. 9	1926 Aug. 28	*Rabbit* 28	1,865	do	do	E 7. AAB	0.585 gm	4	714 gm	2,857 gm	Resin acid	Sick	Died.
9	do	29	1,635	do	do	E 7. AAB	0.292 gm	4	357 gm	1,428 gm	do	do	Recovered.

[a] Per hundredweight of animal.

[b] Meat of sheep fed.

EXTRACTING AND FRACTIONATING THE PLANT

Eight of the lots of plant collected for use in this study were submitted to extraction and fractionation processes, and inasmuch as the procedure was similar in every case a composite description of the procedure followed is presented here, the various fractions being distinguished by a lot number in order to insure clearness. The fractions are further grouped according to the chemical character of the substances present so that comparison may be made between various fractions and between plants from different collections.

In general, the fresh plants were subjected to the following process to extract the soluble constituents and to fractionate the extracts so obtained: The fresh plant was hashed and placed in strong alcohol in a percolator. After two days' maceration the plant was extracted with alcohol by intermittent percolation; the alcohol was removed from the percolate by distillation at atmospheric pressure and was returned to the percolator. The residue from the distillation was mixed with water and redistilled to remove the last traces of alcohol and also to drive off the volatile oil which was recovered.

The residue so obtained consisted of a greenish, insoluble mass of lardy consistency, and a watery solution. The water solution was siphoned off, and the insoluble residue was repeatedly extracted with boiling water as long as it yielded soluble matter. The combined water extracts furnished one fraction.

The insoluble matter was next treated with hot 1 per cent sodium-carbonate solution which dissolved out a resin acid fraction, leaving undissolved what was termed the "lipoid" fraction. The latter was fractionated in various ways, which are described in connection with the testing of the fractions themselves.

The water-soluble materials were fed in aqueous solution. The resin acid was fed in sodium-carbonate solution, and other materials were emulsified in water or in sirup by means of acacia.

FRESH LEAVES

Leaves from plants collected at Woodmont, Va., were fed to two guinea pigs October 7 to 22, 1921, a total of 10 doses, averaging 21.75 gm. per dose, being given. The animals had access to oats during the experiment. Both were made sick, and one died 18 days after the last feeding. Post-mortem examination showed liver and kidneys pale, lungs congested, and congestion in the stomach and colon. The other animal survived.

Sheep 5 was fed by balling gun nine doses of fresh leaves collected at Woodmont, Va. From June 20 to 30, 1925, this sheep was fed 5,123 gm. of fresh plant, equivalent to 15 pounds per hundredweight of animal. This feeding produced no effect on the animal.

TOTAL WATER-SOLUBLE NONVOLATILE CONSTITUENTS

The material of plant lot E 5, collected in Beecher City, Ill., and shipped to Washington preserved in chloroform water, was allowed to stand in storage during the winter. It was opened in May, 1921, when the water solution was strained off. The leaves of the plant were stripped from the stems, hashed in a meat chopper and pressed out, the strained juice being added to the water solution. Half of

the solution was distilled from a copper still until volatile substance, other than water ceased to appear in the distillate. The nonvolatile residue (E 5. BAB) was collected and used for a feeding test. This was a limpid, faintly greenish-brown, acid liquid, of bitter, saline, and highly unpleasant taste. It was colored deep green with ferric chloride, even after the removal of possible tannins with gelatine solution. It yielded no alkaloid test with Mayer's solution. Alcohol precipitated a large amount of gray, flocculent matter (inulin). The whole residue was concentrated to 2,700 c. c. by evaporation.

Sixteen doses of 162 c. c. of this solution, corresponding to 2.5 pounds of fresh plant per hundredweight of animal, were drenched into sheep 650 from July 16 to July 31, 1921. The total dosage was 2,592 c. c., equivalent to 40 pounds of plant per hundredweight. The sheep was not affected by this treatment.

VOLATILE OIL

Volatile oils were obtained in concentrating alcoholic extracts of Eupatorium. Oils obtained from green plant gave a positive test for tremetol; a sample from dry plant was negative. An emulsion containing 5 gm. of oil obtained from lot E 7 was drenched into sheep 779 September 25, 1923, at 1 p. m. The animal was observed during the afternoon but showed nothing abnormal. It was found dead, however, on the following day at 6.30 a. m.

A post-mortem examination conducted during the morning by G. T. Creech, of the pathological division, revealed hemorrhagic areas on the endocardium; the liver pale and degenerate; the kidneys degenerate; hemorrhages in the lower end of ileum and duodenum; and thyroid and thymus congested. There was much fluid in the abdominal cavity. Certain of these abdominal conditions were considered to be of longer standing than the present feeding would account for, and possibly were the result of earlier experiments made on this sheep.

The volatile oil, emulsified in sirup with acacia, was fed to cats 17 and 19. On November 26, 1923, cat 17 received 0.45 gm. of oil and November 27, 0.60 gm. On November 26 cat 19 received 0.30 gm. and November 27, 0.60 gm. of oil. Both animals survived the doses and showed no abnormal effects from them.

From April 15 to 30, 1924, sheep 782 was given 13 daily doses of 1 gm. each of oil, emulsified in sirup with acacia. The total dosage, equivalent to 32.75 pounds of green plant per hundredweight of animal, produced no effect.

From these experiments it is apparent that the essential oil, while toxic in large doses, is not the constituent responsible for trembles.

WATER- AND ALCOHOL-SOLUBLE CONSTITUENTS

LOT NO. E 1

The material consisted of 1,805 gm. of green plant that had been hashed and put into alcohol November 17, 1916, by W. N. Berg, then of this laboratory, who turned the specimen over to the writer in 1918. October 15, 1918, it was packed into two glass percolators and exhausted with strong alcohol. The alcohol was removed from the percolate by distillation, leaving a brown, aqueous solution on

the surface of which was an oily layer. A resinous precipitate was distributed throughout the aqueous solution. The whole was extracted with petroleum ether (E 1. AB). The water residue was filtered from the precipitated resin and tested on guinea pig 46, in 10 c. c. doses, equivalent to 65.64 gm. of green plant. Five doses were given from November 21 to November 27. The animal showed no abnormal effects from these doses. It was killed for autopsy, which revealed a slight gastroenteritis.

LOT NO. E 2

Four thousand four hundred and thirty-two gm. of dried whole plant were ground to a No. 20 powder and extracted with strong alcohol. The solvent was distilled from the percolate and the resinous residue extracted with boiling water. The filtered water solution was evaporated to convenient bulk. It responded to Mayer's test for alkaloids; consequently it was made alkaline with sodium hydroxide and shaken out first with chloroform which removed very little, and then with amyl alcohol which removed a basic substance.

VOLATILE, WATER-SOLUBLE MATERIAL

The distillate from the water solution of E 5 was cohobated until its volume was reduced to 6 liters. It was treated with a slight excess of sodium hydroxide and submitted to steam distillation until pure water alone came over. The distillate (E 5. BAAA) bore the characteristic odor of the plant, but only minute quantities of oil separated. It did not react with ferric chloride. Fourteen doses of it drenched into sheep 639 resulted in no other effect than a slight catharsis. The alkaline residue (E 5. BAAB) was evaporated to dryness when it weighed 48 gm. Half of it was dissolved in water, neutralized with acetic acid, and fed to sheep 631 in divided doses. The sheep remained normal.

The extracted water solution (E 2. ABC) was fed to sheep 539. Four doses estimated to represent 3 pounds of green plant each and 4 doses estimated to represent 4.5 pounds of green plant each, a total of 30 pounds, fed to this animal, produced no ill effects.

LOT NO. E 5

The press cake (see p. 560) was extracted similarly with alcohol. The resinous residue obtained was fractionated with sodium-carbonate solution which dissolved the remaining water-soluble constituents as well as a resin acid. The testing of this fraction is described under the heading "Resin acids."

LOTS NOS. E 6, E 7, E 9, AND E 10

E 6, E 7, E 9, and E 10 yielded similar water solutions. E 6 B was fed to sheep 6 in 100 gm. doses. Five daily doses each, equivalent to 8 pounds per hundredweight given to the animals, produced no effect. The water solution gave evidence of the presence of a glucoside and responded to Mayer's test. Acetic acid was present, and levulose, probably resulting from the hydrolysis of inulin, was detected. The water solution from E 7 was tested for tremetol with negative results.

ALKALOID

The amyl-alcohol solution containing the alkaloidal substance extracted from E 2. ABC was treated with dilute hydrochloric acid which extracted the base. A quantity of this, estimated to be equivalent to 24.4 pounds of green plant, was fed to sheep 534 August 19, 1919, and did not affect the animal.

ALCOHOL-SOLUBLE, WATER-INSOLUBLE CONSTITUENTS

FATS, RESINS, RESIN ACIDS, ESTERS

LOT NO. E 1

Half of the resinous precipitate was emulsified in mucilage or Irish moss and given to guinea pig 55 in a dose equivalent to 900 gm. of the green plant. The animal died during the night after showing symptoms of depression. The autopsy was negative.

Thereafter all resinous fractions were treated with sodium-carbonate solution before testing on experimental animals.

The remaining half of the resinous fraction was treated with sodium carbonate solution which dissolved a large portion of it. This fraction was given to guinea pig 57 in doses beginning December 13, 1918. The animal became depressed, rapidly lost weight and died on December 31 showing symptoms of respiratory paralysis. An autopsy showed the animal greatly emaciated; the organs, however, appeared normal.

LOT NO. E 2

A similar fraction containing resin acids (E 2. AAA) was dissolved in sodium-carbonate solution and fed to sheep 550 in 33 doses from July 5 to July 13, 1919. The quantity fed was increased from 1 to 15 gm. per dose. An equivalent of 35.31 pounds of green plant fed to the sheep during this period produced no effect.

LOT NO. E 5

The resin-acid fraction from this extraction (E 5. AAA) was dissolved in sodium-carbonate solution and fed to sheep 622 from July 22 to August 4, 1921, in doses equivalent to 2.5 pounds per hundredweight. Fourteen doses were administered, equal to 35 pounds of green plant. There was a slight but definite depression of the pulse but no other abnormal symptom was observed and the animal recovered.

LOT NO. E 7

The homologous fraction (E 7. AAB) was fed to sheep 6 from August 3 to August 10, 1926, in seven daily doses, each equivalent to 7.5 pounds of green plant per hundredweight of animal, or a total of 52.5 pounds. There was no effect from this feeding.

A series of feedings of this fraction were made to 6 guinea pigs and 2 rabbits. The results are tabulated in Tables 3 and 4. Of the 6 guinea pigs, 5 were made sick and 4 of them died.

TABLE 3.—*Quantities of resin acids fed to rabbits and guinea pigs, 1926*

Date	Guinea pig No.						Rabbit No.	
	297	298	299	300	301	302	28	29
	Mgm.	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*	*Mgm.*
Aug. 9	39	78	136.5	58.5	117	156	476.15	238.07
Aug. 11	39	78	136.5	58.5	117	.....	476.15	238.07
Aug. 20	78	.....	273	117	234	.....	952.3	476.15
Aug. 28	.....	.....	273	117	.....	.....	952.3	476.15
Sept. 2	.....	.....	273	117	.....	.....	.....	.....

TABLE 4.—*Weights of guinea pigs and rabbits fed resin acids, 1926–27*

Date	Guinea pig No.						Rabbit No.	
	297	298	299	300	301	302	28	29
1926:	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*
Aug. 9	460	430	545	470	510	560	1,865	1,635
Aug. 11	480	450	550	490	510	Dead.	1,845	1,595
Aug. 14	470	400	580	495	500	.....	1,815	1,595
Aug. 16	435	355	575	495	500	.....	1,880	1,605
Aug. 19	400	Dead.	595	525	520	.....	1,870	1,610
Aug. 26	330	.....	625	545	Dead.	.....	1,895	1,630
Aug. 28	310	.....	625	540	.....	.....	1,895	1,605
Sept. 2	Dead.	.....	640	525	.....	.....	1,880	1,670
Sept. 29	.....	.....	.....	.....	.....	.....	1,550	1,790
Oct. 7	.....	.....	.....	.....	.....	.....	1,380	1,740
Oct. 11	.....	.....	.....	.....	.....	.....	1,305	1,715
Oct. 16	.....	.....	.....	.....	.....	.....	1,115	1,690
1927:								
Jan. 7	.....	.....	.....	.....	.....	.....	.....	1,450

Guinea pig 302 may have succumbed to a traumatic pneumonia. The three other pigs remaining, which died from the dosage, all showed the same symptoms of depression. The autopsies revealed anemia, great emaciation, enlarged liver and spleen. The urine collected at autopsy was usually pale, cloudy, acid, and contained albumin. Acetone, dicetic acid, and reducing substances were not found. The two rabbits fed were made sick. Rabbit 28 died October 17, after losing a total of 750 gm. of its original weight. The post-mortem examination revealed emaciation and anemia. Urine collected at autopsy was acid and contained albumin. Sugar and acetone bodies were absent.

Rabbit 29 was observed for five months and was finally discharged January 7, 1927, when he had lost a total of 185 gm. of his original weight. This animal survived the treatment, but appeared to be permanently affected, inasmuch as he failed to gain weight or to recover from depression, although more than four months had elasped since the last feeding of resin acids.

NONACIDIC CONSTITUENTS OF THE RESINOUS FRACTION

FATS, WAXES, ESTERS, AND "LIPOID FRACTION"

LOT NO. E 1

The portion of the resin not soluble in sodium carbonate was emulsified in 50 per cent alcohol and given to guinea pig 70 January 2, 1919. The animal became depressed, lost 26 per cent of his

original weight, and finally died January 14. Post-mortem change was so far advanced that no conclusions could be drawn from the autopsy.

LOT NO. E 2

A similar fraction (E 2. AAB) was emulsified in water and drenched into sheep 522 in two doses. No effect was observed.

LOT NO. E 5

The corresponding fraction (E 5. AAB) was emulsified in water with acacia and seven doses of 65 c. c. each, equivalent to 2.5 pounds of green plant per hundredweight of animal, were given to sheep 625 from August 30 to September 5, 1921. The sheep developed a positive case of trembles. Feeding was discontinued and the animal slowly recovered. Another portion of the same fraction was given in seven doses of 66 c. c. (2.5 pounds per hundredweight) to sheep 650 from September 6 to September 13. The animal developed a positive case of trembles. She was killed for autopsy September 19. The post-mortem examination showed some slight congestion in the lower ileum, liver pale and yellowish, and the cortex of the kidney dark. A sample of blood drawn from the left jugular vein immediately after death was tested for acetone bodies, with a positive result.

Specimens of urine obtained from both sheep 625 and sheep 650 contained acetone, and the odor of that ketone was noticeable on the exhaled air in both cases.

LOT NO. E 6

The corresponding fraction from this extraction (E 6. AAA) was not fed as such but was divided by means of solvents into several portions, as follows:

Petroleum ether extracted	0.768 per cent of green plant (E 6. 3AB)
Benzene extracted	0.36 per cent of green plant (E 6. 4AB)
Alcohol extracted	0.098 per cent of green plant (E 6. 5AC)

The alcohol extract was divided, and a small portion of it was extracted successively with ether and chloroform, as follows:

Ether extracted	0.062 per cent of green plant (E 6. 5AB)
Chloroform extracted	0.033 per cent of green plant (E 6. 6AB)

The material extracted by the solvents was given to sheep and guinea pigs.

The petroleum-ether fraction was emulsified in water with acacia and 12 doses, each equivalent to 2.5 pounds of green plant per hundredweight of animal, were drenched into sheep 677 from July 10 to July 21, 1922, with no effect on the animal. Three doses of 1 gm. each (emulsified) were given to guinea pig 197 from December 13 to December 19, 1921. The animal was made sick but recovered.

A series of 21 doses of the fraction emulsified in water with acacia were given to guinea pigs 199 and 200 from March 8 to April 1, 1922, the doses being gradually increased from 2 to 5 gm. during the interval. Guinea pig 200 was made sick and died April 9. Pig 199 showed no abnormal effect from the feeding and survived. Guinea pig 197 which received 3 gm. doses of this fraction from December 13 to December 19, 1921, was made sick, but eventually recovered. This fraction gave a faintly positive test for tremetol.

The benzene fraction was emulsified in water with acacia and fed to guinea pigs 199 and 200 from January 26 to February 16, 1922. Each animal received 14 doses of 0.3 gm. corresponding to 78.3 gm. of green plant, or a total of 1,096.2 gm. of green plant for the entire period. Neither animal was affected by this treatment; both gained weight and appeared perfectly healthy during the experiment. The fraction was emulsified and fed to sheep 674 from July 15 to July 25, 1922, in eight doses of 2.5 pounds and two doses of 5 pounds of green plant per hundredweight. The total dosage, 30 pounds, did not affect the animal. This fraction did not respond to the test for tremetol.

The ether fraction was emulsified in water with acacia and fed to guinea pigs 201 and 202. Each animal received seven daily doses of 0.1 gm., equivalent in all to 200.7 gm. of green plant, from February 20 to February 28, 1922. Neither animal was affected by the material given.

The alcohol fraction was emulsified in water with acacia and fed to sheep 679 in 12 daily doses of 2.5 pounds per hundredweight from July 11 to July 22, 1922. On July 24 and 25 the doses were doubled so that the sheep received a total of 40 pounds per hundredweight. The sheep showed some slight symptoms of colic on July 22 and 23 but otherwise remained normal. The alcohol fraction was also fed to guinea pigs 201 and 202 in four doses totaling 0.75 gm. of fraction, equivalent to 1,405 gm. of green plant. Sheep No. 202 was not affected but sheep No. 201 became sick and died one month after the last feeding. The post-mortem examination revealed anemia, a pale liver, and pale kidneys. The fraction did not respond to the test for tremetol.

A mixture of the petroleum-ether and benzene fractions was made in equivalent proportions, and this mixture, equal to 15 pounds of green plant per hundredweight of sheep, was emulsified in water with acacia and fed to sheep 679 from July 27 to August 1, 1922, in immediate succession to feedings with the alcohol extract. (See above.) There was no effect from this treatment.

LOT NO. E 7

The residue obtained by distilling the solvent from the alcoholic percolate of this plant consisted of mixed fats, resins, and water solution. It was so difficult to filter that it was extracted with benzene, which dissolved the major portion of the water-insoluble matter present. The benzene solution was washed with several portions of aqueous sodium carbonate to free it from acidic constituents, separated from the water solution, and the benzene distilled off. The residue was a deep-green mass of the consistency of lard. It weighed 664 gm. and constituted 2.04 per cent of the green plant extracted. Four daily doses of an emulsion of this fraction were given in drenches to sheep 707. The animal received a total of 18.4 pounds of green plant equivalent per hundredweight from March 13 to March 16, 1923. The animal developed trembles and died during the night of March 18. The post-mortem examination revealed the lungs congested, light-colored areas in the liver which had the appearance of fatty degeneration, and the spleen pale. The other organs and the musculature were pale. Post-mortem change was marked. The

meat from this carcass was fed to cats 17, 18, and 19 from March 19 to March 28, 1923, but did not affect them.

Two doses of this fraction were given to guinea pig 213, the first February 28 and the second March 1, 1923. Each dose consisted of 0.2 gm. of the fraction and was equivalent to 10 gm. of green plant. The animal remained normal until March 10, when symptoms of depression appeared. These became progressively worse and the animal died March 23. The autopsy showed some congestion of the lungs, spleen pale and brownish, liver dark, and other organs normal. Urine taken from the bladder did not contain acetone.

LOT NO. E 9

A similar fraction (E 9. A) was emulsified in sirup with acacia and four doses of 27.4 gm., equivalent in all to 16 pounds of green plant per hundredweight, were given to sheep 782 from February 9 to February 12, 1925, without affecting the animal. The same fraction was fed twice to sheep 4 without producing trembles; from April 1 to April 9, 1925, this sheep received eight daily doses of 15 gm., a total of 32 pounds of green plant per hundredweight, and from April 22 to April 25, 1925, the sheep received four daily doses of 30 gm., equivalent in all to 32 pounds of green plant per hundredweight. Various fractions of this extract were also fed and are described below. In no case were symptoms observed and it was concluded that the original plant was nontoxic or that the process of extracting the soluble constituents had in some way altered the toxic principle.

Inasmuch as E 7 was processed in the same way and was toxic, it does not seem probable that the last hypothesis is correct. This fraction was later tested for tremetol and was found to contain a very small quantity of the toxic substance.

LOT NO. E 10

The corresponding fraction from this lot was obtained by treating the residue that remained, after the alcohol had been distilled from the percolate, with boiling water as long as soluble material was extracted. The insoluble material was then extracted with boiling 50 per cent alcohol as long as anything could be extracted. The insoluble matter was labeled E 10. AAA. It gave no test for tremetol. Twelve daily doses of this fraction, emulsified in sirup with acacia, and consisting of 27 gm. each, equivalent to 3 pounds of green plant per hundredweight, were given to sheep 5 from March 22 to April 3, 1926. The sheep was not affected. The material dissolved in 50 per cent alcohol was recovered by distilling off the solvent. This fraction responded to the test for tremetol. It was emulsified in sirup with acacia and given to sheep 5. From March 4 to March 6, 1926, the sheep received three daily doses of 7 gm., equivalent to 1.65 pounds of green plant per hundredweight. From March 7 to March 18, 1926, the sheep received nine daily doses of 10.56 gm., equivalent to 2.5 pounds per hundredweight. No effect was apparent, although the total dosage was equivalent to 27.5 pounds of green plant.

From April 12 to April 15, 1926, the same sheep was given four daily doses of 21.12 gm. each, corresponding to 5 pounds of green plant per hundredweight, or a total of 20 pounds. The sheep remained normal until April 16, when he began to tremble. He became pro-

gressively worse and died April 22. During the sickness several samples of urine and blood were obtained. On April 16, when trembling first appeared, the sheep was not excreting acetone bodies, but on the following morning there was an acetone odor on the breath and the urine contained a quantity of this ketone. From that day onward the animal excreted acetone continuously by the lungs and kidneys. At autopsy the only marked abnormality was apparent degeneration of the liver. The carcass had a strong odor of acetone. Blood and urine, collected at autopsy, were tested and found to contain acetone. In the urine the proportion of acetone was 24.32 mg. per 100 c. c. The blood sugar was markedly high during the sickness. On March 22, when the sheep appeared to be normal, a sample of blood drawn from the left jugular vein was subjected to analysis and the content of blood sugar was determined as 0.104 gm. per 100 c. c. Blood drawn similarly April 16 when the sheep was trembling gave an average of 0.168 gm. per 100 c. c. for two determinations. At this time feeding with the Eupatorium extract was discontinued and the sheep was given oats. Another sample of blood was taken April 22 from which a blood sugar content of 0.138 gm. per 100 c. c. was determined.

The remainder of the extract that had proved fatal to sheep 5 was treated with boiling 30 per cent alcohol in successive portions as long as soluble matter was dissolved. The insoluble residue (E 10. AABB) which gave no test for tremetol, was tested on sheep 6. Five daily doses of the emulsified material, equivalent to 7.5 pounds of green plant per hundredweight of sheep per dose, were given from May 8 to May 13, 1926. The total dosage was 37.5 pounds. There was no effect from this feeding.

The results obtained from feeding experiments with the "lipoid" fraction indicated clearly that the toxic principle responsible for trembles was contained in that fraction. Failure to produce poisoning in sheep with the extracts made from lots E 6 and E 9 was apparently due to their small content of tremetol. Lot E 6 was collected at Woodmont, Va., in a district where milk sickness had not been observed, so far as the writer knows. It is possible that plants from this locality are innocuous. Lot E 9, however, was collected in two places in Illinois from which very toxic plants were obtained at other times. Lot E 2 failed to yield a toxic lipoid fraction; this material was dried plant, however, and was not expected to be very toxic.

HYDROLYSIS OF THE LIPOID FRACTION

LOT NO. E 7

Failure to obtain crystalline substances by chemical treatment of the fraction led to the decision to hydrolyze it with alcoholic potassium hydroxide. Accordingly this was done. A quantity of the toxic fraction was boiled under reflux with 5 per cent alcoholic potash until hydrolysis was complete; the alcohol was then removed and the bright-green residue was treated with water in which the soaps dissolved and emulsified the insoluble matter. This mixture was then repeatedly extracted with ether, which slowly removed a yellowish mass that was obtained by evaporating the solvent. The residue is described below as the "sterol" fraction. The soaps were decomposed with hydrochloric acid, and the precipitated mass was filtered off. The tests made on this material are described under the heading

"fatty acids." The filtrate was further extracted with ether to recover the water-soluble acids; the ether was shaken with sodium-carbonate solution, which took out a small quantity of acetic acid. Following the ether extraction the water solution was evaporated to small volume, and glycerine was detected in the residue.

THE FATTY ACIDS

LOT NO. E 7

The precipitate containing the fatty acids was washed with water, and a portion was emulsified in acacia and water for feeding. From April 3 to April 11, 1923, sheep 708 received four doses of this emulsion, each equivalent to 4 pounds of green plant per hundredweight, or a total of 24.3 gm. of the fraction.

On April 4 the sheep gave birth to a normal lamb. The animal was rested for 5 days before the experiment was resumed. There was no toxic effect from this feeding.

THE STEROL FRACTION

LOT NO. E 7

The material extracted by ether from the mixture of hydrolytic products consisted of a yellowish, partly crystalline, unctuous mass which had an agreeable aromatic odor, somewhat resembling that of nutmeg. Three daily doses of this fraction each containing 10.72 gm., equivalent to 4 pounds per hundredweight, were fed in emulsion to sheep 708 from April 16 to April 18, 1923. On April 19 the animal was unable to stand without trembling. The symptoms of trembles became worse, and the animal died Sunday morning, April 22. By the following day post-mortem change was so advanced that no conclusions could be drawn from autopsy.

The suckling lamb of this ewe, sheep 709, had received the milk from the mother during the feeding and the subsequent disease period. At the beginning of the experiment the lamb was healthy and normal. On April 21, two days after the appearance of trembles in the mother, the lamb also showed symptoms of trembles. These developed rapidly until the lamb was unable to stand. After the death of the mother the lamb was fed cow's milk for several days. During this period no improvement occurred. A specimen of urine was obtained April 26. This was acid and contained acetone. The lamb was also excreting acetone by the lungs. The lamb was killed for autopsy April 28 and the post-mortem examination was immediately made. The liver appeared discolored and the kidneys were degenerated. The other organs appeared to be normal.

This experiment definitely located the toxic principle in the sterol fraction and there arose the problem of separating it from the other sterols present. A portion was submitted to steam distillation, but only a very small quantity of volatile material was obtained. The main portion of the sterol fraction was dissolved in hot 85 per cent alcohol and allowed to cool when a considerable quantity of needle-shaped crystals separated out. These were collected by filtration and the filtrate was twice concentrated, each time yielding a crop of the same substance. These crops were united and recrystallized from 85 per cent alcohol, when the substance was obtained pure.

The mother liquors yielded an impure crop of this substance that was contaminated with oily matter.

The mother liquors from which the crystalline sterol was obtained were concentrated by evaporation, but nothing further could be made to crystallize from them. The solvent was removed and left a viscous, oily residue of aromatic odor. The larger portion was soluble in petroleum ether. The soluble matter was labeled E 7.6 A, the insoluble E 7.5 AB.

THE CRYSTALLINE STEROL

The crystalline sterol was a mass of soft, white needles melting at 148 to 149° C. and soluble in the ordinary organic solvents. Elementary analysis indicates that the formula is $C_{18}H_{30}O$. It does not give the characteristic reactions of phytosterols. A quantity of this substance was emulsified in water with acacia. Four daily doses each containing 1.2 gm. of the substance, equivalent to 4 pounds of green plant per hundredweight of sheep, were fed to sheep 779 from July 3 to July 7, 1923, but produced no effect on the animal. A second feeding of this mixture was made between August 28 and September 5, 1923, when the same sheep received 6 daily doses of 2.5 gm. and one of 2 gm., or a total of 54.4 pounds equivalent of green plant. There was no effect from these feedings.

LOT NO. E 9

A similar fraction from another lot (E 9. AAD), which consisted largely of the sterol, was fed in varying doses from June 24 to July 5, 1924, to sheep 782. This animal received in all 9 doses containing a total of 33.4 gm. of the fraction, equivalent to 63.8 pounds of green plant per hundredweight. There was no effect from this feeding.

These findings showed that the toxic principle was to be sought for in the other two fractions of the original sterol fraction, the portion soluble in petroleum ether, and the small quantity of material insoluble in that solvent.

THE PETROLEUM-ETHER INSOLUBLE FRACTION

This fraction labeled E 7.5 AB was a thick, brownish noncrystalline mass which weighed 8 gm. Nothing crystalline could be prepared from it. It was emulsified in water with acacia and fed in four doses of 1 gm. each to sheep 779 from August 2 to August 6, 1923, and in a dose of 3 gm. August 14. There was no effect from the feeding.

THE PETROLEUM-ETHER SOLUBLE FRACTION

The experimental feedings, described above, which showed the lack of toxicity of all other fractions of the original sterol fraction made it evident that E 7.6 A contained the toxic principle. Efforts were then directed toward separating this fraction into its constituents, which were mainly tremetol, with some crystalline sterol, coloring matters, probably carrotin, and perhaps other unknown substances. A portion of the crystalline sterol was removed by cooling the fraction to a low temperature and filtering it from the separated crystals. Other methods of separation were tried but none were successful, and finally it was decided to distill the fraction under very diminished pressure and to test the distillates.

Three fractions were obtained, upon vacuum distillation, as follows:

Distillate I, 188 to 192° C. (15 mm.) 15 gm.
Distillate II, 192 to 256° C. 10 gm.
Residue.

The distillates were united and again distilled. Four fractions were obtained as follows:

Distillate I, 166 to 170° C. (15mm.) 5 gm. Tremetol test, negative.
Distillate II, 170 to 205° C. (12 mm.) 10 gm. Tremetol test, positive.
Distillate III, 205 to 235° C. (12 mm.) 5 gm. Tremetol test, negative.
Residue very small.

The temperatures at which the second distillates were obtained indicate that there had been some decomposition during the first distillation.

All the distillates were thick, oily liquids; the higher boiling fraction on cooling deposited a small quantity of crystals.

Distillate II was injected intraperitoneally into three guinea pigs June 14, 1923. Pig No. 236 received 0.2 c. c., pig No. 211 received 0.3 c. c., and pig No. 239 received 0.5 c. c. All were made sick and No. 239 died during the night of June 15–16. The post-mortem examination revealed a considerable yellowing of the liver and pale kidneys, the other organs being normal. Guinea pigs 211 and 236 were depressed for several days but finally recovered.

Four grams of this fraction were mixed with a little cottonseed oil (to facilitate emulsification), emulsified in water with acacia, and fed in four daily doses of 1 gm. each to sheep 779, from July11 to July 14, 1923. There was no effect from this feeding.

A similar distillate from lot E 8 fed to sheep 782 from January 2 to January 5, 1924, in four doses of 1.25 gm. each (emulsified) produced no effect on the animal.

The remaining vacuum distillates and the residue obtained in the first distillation were fed separately in large doses to this sheep, but in no case was the animal affected. It seemed likely, therefore, that the toxic substance was altered by the distillation.

A small quantity of the undistilled petroleum-ether-soluble material, calculated to be equivalent to 31.34 pounds of green plant, but probably equivalent to much less on account of various losses incidental to chemical processing, was emulsified and fed to sheep 779 in two doses on September 12 and 13, 1923. This treatment appeared markedly to depress the animal, but no trembling was observed, and the feedings could not be continued on account of lack of material.

TREMETOL

Fractions E 5. AAB, E 7. 3A, and E 10. AAB, which consisted in part of tremetol and fractions E 7.4A and 7.5A, which were impure tremetol, were fed to sheep 625, 650, 707, 5, 708, and 779. All the sheep were affected and the first five developed characteristic symptoms of trembles; sheep 625 recovered and the remainder died. Sheep 709, a suckling lamb that received the milk of sheep 708 while the latter was sick with trembles, also developed characteristic symptoms of the poisoning.

Many feeding tests have been made on guinea pigs, but these animals have not reacted in any uniform fashion to the doses given. Highly purified tremetol, from a lot used for chemical analyses and

molecular-weight determinations, was injected intraperitoneally into 4 guinea pigs July 8, 1926. Pigs 293, 294, 295, and 296 received respectively 0.2 c. c., 0.1 c. c., 0.15 c. c., and 0.3 c. c. All were made sick, pigs 294 and 295 died in 7 days, and the post-mortem examination,[4] disclosed the nutmeg liver commonly found in trembles cases. The remaining animals that received the larger doses survived. On July 27 these were again injected intraperitoneally, pig 293 receiving 0.25 c. c. and pig 296 receiving 0.3 c. c. Both were made sick. Pig 296 showed very severe symptoms, lost considerable weight, and died August 29.

The autopsy revealed emaciation, anemia, and some enlargement of the liver. The urine was acid and contained albumin, but no acetone bodies. At first, pig No. 293 did not appear greatly affected. He became depressed and finally lost weight and was found dead September 15, seven weeks after the last injection. Autopsy showed emaciation, and anemia. The liver was pale and contained several large, yellowish areas, the kidneys were pale, and the spleen was enlarged and dark. There were three small healed ulcers on the mucosa of the ileum. No odor of acetone was present.

The experimental evidence indicates that tremetol is the substance that causes the disease known as trembles in cattle, horses, and sheep. On account of the small quantity of pure tremetol available it was impossible to conduct confirmatory experiments on large animals during this investigation. Final proof of the causal relationship of tremetol to trembles, therefore, must be delayed until such experiments can be carried out. Efforts are in progress to prepare a quantity of pure tremetol for this purpose.

FEEDING OF EXTRACTS THAT DID NOT CONTAIN TREMETOL

All other constituents of the plant were fed at one time or another to sheep, guinea pigs, rabbits, and cats. The only other constituents of the plant that produced death or symptoms of poisoning were the resin acids and the volatile oil. Several feedings of the resin acids to sheep 6, 550, 622, and 679 produced no effect except that in the case of sheep 622 a small but definite depression of the pulse rate was observed. Certainly the resin acids are incapable of producing trembles and probably play no rôle in milk sickness under usual conditions.

The resin acids are, however, toxic to rabbits and guinea pigs, when derived from either green or dried plants.

It is very likely that many of the cases produced in guinea pigs and rabbits in experimental feeding with *Eupatorium urticaefolium*, green and dried, and with extracts made from it, have been the result of poisoning by the resin acids rather than by tremetol. This would account for the toxicity of the dried plant and its extracts to laboratory animals as reported by several investigators, a fact which seemed contrary to the general experience that dried plant would not produce trembles in cattle and sheep when fed in moderately large doses. Drying the plant apparently does not greatly diminish the toxicity of these resin acids.

None of the remaining constituents of *Eupatorium urticaefolium* was observed to be harmful to the animals experimented with even in the very large doses sometimes fed.

[4] Conducted by W. S. Gochenour, to whom the author wishes to express his appreciation.

GLUCOSIDES

Aqueous extracts that contain the glucosidal substance present in richweed fed to several guinea pigs and to sheep 539, 596, 650, and 6 produced no effect on these animals. This fact considered in conjunction with other evidence as to the character of the toxic principle seems to indicate that this glucoside is not toxic and bears no relationship to trembles.

ALKALOIDS

The basic substances extracted from lot E 2 were fed to sheep 534. The bases were also present in the aqueous extracts fed as described in the preceding paragraph. No evidence of toxicity was obtained as a result of these feedings, and it is apparent that the basic constituents of richweed are not responsible for the toxicity of the plant.

ALUMINUM PHOSPHATE

No feedings with aluminum phosphate were made. The extensive feeding experiments reported by Wolf, Curtis, and Kaupp (*22*), the abandonment of the aluminum hypothesis by Moseley (*13*), who originated it, the absence of anything in the pharmacology of aluminium compounds that supports the hypothesis, and the discovery of an aluminium-free substance that does produce trembles, all obviate the necessity of experimental work with this compound.

DRIED AND GREEN PLANTS

Lots E 2 and E 8 were specimens of dried *Eupatorium urticaefolium* that had been collected in the neighborhood of Beecher City, Ill., in 1915, and dried and stored, one for 4 and the other for 8 years before being used in this investigation. No cases of trembles were produced by extracts of these specimens. Eight lots of green plant were used, 4 from Virginia and 4 from Illinois. Of the Virginia collection, 1 was extracted and fed only to guinea pigs, 2 others were fed in substance, and the fourth lot was extracted and fractionated and the fractions fed to sheep. None of these feedings produced cases of trembles. Of the 4 lots of green plant obtained from Illinois, 3 were extracted and produced trembles in sheep; the fourth lot was extracted and fractionated but no cases of trembles were obtained from the feeding of this material. Green plant packed in milk cans and preserved in chloroform water furnished the most active extracts used; other samples of green plant that were mailed to Washington from Illinois as soon as they were collected and which were received within 3 or 4 days and immediately placed in alcohol, were of diminished toxicity.

The color reaction for tremetol has been applied also to specimens of green and dried plants. A sample of the plant collected at the time the material of lot 5 was gathered in Illinois, September, 1920, was shipped in a gunny sack to Washington and was dried in the laboratory. After six years' storage there a portion of it was submitted to the test for tremetol with negative results.

Inasmuch as this plant came from a lot that was very toxic, as demonstrated by feeding tests, the failure to get a positive test for tremetol is quite conclusive evidence that drying destroys this toxic principle. Another sample of dried plant, collected at Woodmont, Va., in June, 1921, and immediately dried in the shade, was tested

after five years' storage in the laboratory. This sample was also negative when tested for tremetol.

Specimens of fresh plant from Arlington County, Va., were tested during the summer of 1926. Several plants collected at different times at one place in Woodmont reacted positively to the tremetol test. A number of other specimens, collected at a point about one-half mile from the first lot, and at different times, gave either a faint or a quite negative reaction to the tremetol test.

MILK SICKNESS, TREMBLES, AND ACIDOSIS

Since the first publication on milk sickness by Drake (*4*) reference has been repeatedly made to a peculiar odor on the breath and eructations of milk-sickness patients and animals with trembles, and to the marked odor observed about corpses and carcasses of victims of the poisoning.

Drake says, "The breath is peculiarly disgusting, even loathsome," and in fact this symptom so uniformly accompanied the malady that it was considered pathognomonic by many physicians.

Walsh (*20*) suspecting that the foul odor was due to the excretion of acetone by the lungs and recognizing the similarity between certain symptoms of milk sickness and diabetes, diagnosed human milk sickness as acidosis and tested for acetone in the urine. This ketone was found in several specimens.

Jordan and Harris (*9*), who were in consultation with Walsh, obtained specimens of urine from his cases. In two of these specimens acetone was detected; in a third, taken from a mild case from which the patient had previously recovered, no acetone was detected.

From one of the positive urines R. T. Woodyatt, of the University of Chicago, was able to isolate acetone and identify it by means of the paranitrophenylhydrazone.

Walsh (*21*) has reported additional cases of milk sickness in which acidosis was observed.

In the course of the present study a great deal of evidence has been obtained which demonstrates conclusively that an acidosis accompanies Eupatorium poisoning. The odor of acetone has been observed on the breath of each sheep that was trembling and in all such cases in which a test was made the presence of acetone in the urine was demonstrated. In a few cases, acetone was tested for and demonstrated in the blood. Inasmuch as symptoms of severe poisoning appear at least 24 hours before acetone may be detected in the urine or on the breath, it seems that the acidosis is a consequence of the poisoning and not one of the casual factors.

The results obtained in the examinations for acetone are presented in Table 5.

TABLE 5.—*Excretion of acetone by sheep poisoned by richweed*

Sheep No.	Date of trembling	Date acetone appeared on breath	Date acetone was found * in urine	Acetone in blood	Termination of sickness
625	Sept. 4, 1921	Sept. 6, 1921	Sept. 6, 1921	Not tested for	Recovered.
650	Sept. 13, 1921	Sept. 15, 1921	Sept. 15, 1921	Present	Killed for autopsy, Sept. 19, 1921.
709	Apr. 21, 1923	Not noted.	Apr. 26, 1923	Not tested for	Killed for autopsy.
5	Apr. 16, 1926	Apr. 17, 1926	Apr. 17, 1926	Present	Died Apr. 22, 1926.

* In larger amounts than traces.

The effect on the blood-sugar concentration is shown in the case of sheep 5 whose normal blood-sugar content was 0.104 gm. per 100 c. c. On the day when the first trembling was noted the blood sugar had risen to 0.168 gm. This was followed by a fall to 0.138 gm. six days later, due probably to the fact that feeding with the Eupatorium extract was discontinued as soon as symptoms of trembling appeared and remedial measures were begun.

CONCLUSIONS

The chemical and pharmacological data indicate that the constituent of *Eupatorium urticaefolium* which is responsible for trembles is tremetol, $C_{16}H_{22}O_3$. Tremetol has the properties of an unsaturated alcohol and is soluble in fats and fat solvents. It may be secreted in milk and would be found in butter made from such milk.

When tremetol is dissolved in petroleum ether and the solution is floated on the surface of sulphuric acid, a characteristic red color is produced.

A volatile oil and a resin acid are also present in this plant. Neither of these is capable of producing trembles in sheep. The resin acid is toxic to rabbits and guinea pigs. Small doses of the volatile oil appear harmless, but larger doses are dangerous.

Other constituents of the plant include fatty acids, especially acetic acid; a crystalline sterol, $C_{18}H_{30}O$, melting at 148–9° C.; inulin; levulose; an organic base; and a nontoxic glucoside.

When richweed is dried the tremetol content rapidly diminishes and when completely dry the plant is incapable of producing trembles. The dried plant, however, still contains the toxic resin acid and the volatile oil.

Sheep poisoned by richweed or by tremetol develop an acidosis and excrete acetone through lungs and kidneys. The sugar content of the blood is markedly increased. Guinea pigs that were poisoned by the dry plant, with the resin acid, or tremetol, were not observed to excrete acetone.

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